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NATIONAL INSTITUTE OF GENERAL MEDICAL SCIENCES

PHARMACOLOGICAL SCIENCES PROGRAM

JANUARY 1986

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DEPARTMENT OF HEALTH & HUMAN SERVICES

Public Health Service
National Institutes of Health

Memorandum

Date January, 1986

From Pharmacological Sciences Program
National Institute of General Medical Sciences

Subject FY 1985 Annual Report

To Pharmacological Sciences Program Grantees

Enclosed is a copy of the Pharmacological Sciences Program's Annual Report for Fiscal Year 1985. As in the past, we asked many of you to help us with this endeavor which we find to be extremely useful for keeping track of the rapidly advancing research supported under our Program's auspices. Thank you for your participation, and we hope that you find the report as interesting and useful as we do.

We would also like to take this opportunity to inform you that Dr. James Gilliam has retired from the Public Health Service after 26 years of active service. Dr. Paul Velletri joined the Program in December.

Christine K. Carrico

Christine K. Carrico, Ph.D.
Director

Carl A. Kuether

Carl A. Kuether, Ph.D.
Program Administrator

M. Janet Newburgh

M. Janet Newburgh, Ph.D.
Program Administrator

Elizabeth A. O'Hern

Elizabeth O'Hern, Ph.D.
Program Administrator

Paul A. Velletri

Paul A. Velletri, Ph.D.
Program Administrator

Enclosure

PHARMACOLOGICAL SCIENCES PROGRAM

Professional Staff

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Carl A. Kuether, Ph.D.	Program Administrator
M. Janet Newburgh, Ph.D.	Program Administrator
Elizabeth O'Hern, Ph.D.	Program Administrator
Paul A. Velletri, Ph.D.	Program Administrator

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Patricia P. Driscoll	Grants Clerk
Mary Beth Gallagher	Grants Clerk
Mary Catherine Wancheck	Grants Clerk

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Alison Cole, Ph.D.
Gail Handelmann, Ph.D.
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Brian Huber, Ph.D.
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Robert Levine, Ph.D.
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Nancy Ostrowski, Ph.D.
Laura Plunkett, Ph.D.
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Jacqueline Smith, Ph.D.
Barton Weick, D.V.M., Ph.D.
Howard Wheeling, Ph.D.
W. Scott Young, M.D., Ph.D.

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PHARMACOLOGICAL SCIENCES PROGRAM

SCOPE

This report reviews the activities of the Pharmacological Sciences (PS) Program for Fiscal Year 1985.

OBJECTIVES

The Pharmacological Sciences Program supports a broad spectrum of research aimed at providing an improved understanding of the biological phenomena and related chemical and molecular processes involved in the actions of therapeutic drugs and anesthetics. The scope of the program ranges from synthetic chemistry and biochemical studies in molecular pharmacology to controlled studies in man. Much of the research supported by the Program integrates such fields as genetics, endocrinology, chemistry, and computer science with the more traditional areas of pharmacological investigation in an effort to characterize molecular interactions and to understand their expression in man.

ORGANIZATION AND STAFFING

Several staffing changes occurred in the PS Program this year. In December, Dr. Christine Carrico was named Director of the Program. At the same time, Dr. M. Janet Newburgh joined the PS Program as a health scientist administrator with responsibility for grants and fellowships in both the bio-related chemistry and basic pharmacology portions of the Program. In June, Dr. James Gilliam retired after 26 years in the Public Health Service. In December, Dr. Paul A. Velletri joined the Program as a health scientist administrator.

Currently, Dr. Newburgh and Dr. Carrico handle grants and fellowships in the basic and clinical pharmacology areas. Dr. Newburgh also works with Dr. Carl Kuether to administer the bio-related chemistry section. Dr. Elizabeth O'Hern continues to have responsibility for the anesthesia portion of the Program. All program administrators handle training grants in their respective areas.

RESEARCH OVERVIEW

Research supported by the PS Program is divided into three major areas: anesthesiology, chemistry, and pharmacology. In 1985, approximately $5 million was expended for research in anesthesiology, over $35 million for research in chemistry, and about $19 million for research in pharmacology.

A major portion of the PS Program's budget is expended on research in basic chemistry, in recognition of the importance of such effort to the design, synthesis, and structural elucidation of a wide variety of compounds of potential medicinal and/or practical use. Research in chemistry is multifaceted. A few of the key areas include the design and analysis of model compounds that would shed light on the structure and function of an enzyme's catalytic site; the synthesis of specifically designed artificial enzymes; the synthesis and characterization of specific

enzyme inhibitors; ligands that alter nucleic acid function at very specific locations of the genome; and the design and application of new procedures for the efficient synthesis of complex structures, such as antibiotics and anti-tumor agents. One very exciting area of chemical research is the synthesis of nucleotide oligomers that are complementary to key sequences in a viral genome but that also contain a moiety which is capable of reacting covalently with the viral genome to inactivate it. Faster, more efficient synthetic procedures that are being developed for complex molecules are likely to revolutionize the production of a variety of antibiotics and are making it possible to design related structures that have the desired therapeutic effect but that lack shortcomings characteristic of their natural relatives, such as toxicity or difficulty in delivering the drug to the site of action.

Many plants defend themselves from predators and infective or competing agents by producing toxins. From human society's point of view, such toxins can present problems to individuals or livestock who come into contact, or who eat plant materials containing such toxins. The toxins also are of potential benefit to society if they can be used, or modified for use, as pesticides, antifungals, antibacterials, or anti-tumor agents. An important area of research supported by the PS Program is the biosynthesis and characterization of toxins, as well as identification of the factors or conditions that induce their synthesis.

Active areas of research in pharmacology include: the molecular basis of drug action; drug delivery, absorption, distribution, and elimination; drug metabolism; and factors or substances that modify drug action and drug toxicity. Research activity in both basic and clinical pharmacology is supported. Pharmacological research has progressed to the point that much of the progress during the past year has involved studies at the molecular level. The cytochrome P-450 enzyme family is displaying a diversity that is somewhat reminiscent of immunoglobulin families. The mechanisms by which the synthesis of individual forms of cytochrome P-450 is induced, as well as how the multiple forms of this enzyme family arise, are yielding fascinating information via our current knowledge base and the genetic, biochemical, and molecular biological tools currently available to us. These enzymes are interesting not only because of their effects on xenobiotics, but also because they appear to be involved in normal metabolism of cholesterol, fatty acids, prostaglandins, leukotrienes, bile acids, and sterol hormones.

Another focus of considerable pharmacological research effort is hormone-sensitive adenylate cyclase, a system that directly or indirectly is affected by several drugs. This system has turned out to be considerably more complex than previously thought but is yielding to solution and to understanding. One fascinating finding is the similarity among guanine nucleotide-binding regulatory proteins that serve as information transducers for these receptors, as well as their similarity to at least one oncogene product.

Current NIGMS-supported research in anesthesiology is directed toward obtaining the basic knowledge for the development and improved use of safer anesthetic agents. It includes a major effort directed toward a better understanding of the mechanisms of anesthetic action at the molecular, endocrine, and cellular levels. Other major areas of interest are studies of toxic or side effects of anesthesia, the metabolism of anesthetics, and how effects or the metabolism of anesthesia may be altered in specific cases, for example in burned patients or during surgery.

Anesthesia may significantly affect a patient's reaction to treatment. For example, certain common forms of anesthesia alter differentially the intracranial pressure changes that occur in patients with head trauma who are being treated by hyperventilation. Information regarding these effects clearly is important in management of these patients. Other research is directed at understanding toxic side effects of anesthesia and identifying those individuals who have a greater than normal tendency for adverse reaction to anesthesia. For example, one project is seeking a better understanding of the molecular mechanism of malignant hyperthermia and how to determine whether a given individual is susceptible to this condition.

In April, the PS Program sponsored a symposium on "Novel Genetic Approaches in Molecular Pharmacology" as part of the spring meeting of the American Society for Pharmacology and Experimental Therapeutics in Anaheim. The thought-provoking presentations covered a range of contemporary research topics in pharmacological sciences at this well-attended symposium.

The 20th anniversary of the PS Program was celebrated in March with a reception that was attended by Pharmacology Research Associate Program (PRAT) preceptors, as well as present and many former PRAT fellows. Also part of the celebration was a lecture by Dr. Alfred Gilman, a former PRAT fellow, and a poster presentation of the exciting and varied research projects being undertaken by the current PRAT fellows at NIH.

RESEARCH TRAINING

Pharmacological scientists serve important roles in all areas of scientific and health-related endeavors in academia, government and industry. To provide a continuing source of such scientists is the purpose of the training programs of the PS Program. Since 1975, research training in the pharmacological sciences and clinical pharmacology has been supported under the authority of the National Research Service Act. As of 1983, research training in anesthesiology has also come under the auspices of the PS Program. Multidisciplinary predoctoral training programs in the pharmacological sciences are supported by institutional fellowships, as is postdoctoral research training in anesthesiology and clinical pharmacology. Individual fellowships also are available for postdoctoral training in all of the above fields.

The multidisciplinary nature of the institutional predoctoral training programs in the pharmacological sciences provides the student with a combination of skills and techniques necessary to address a wide spectrum of pharmacological problems. Programs are designed to offer students the opportunity to apply knowledge of molecular biology, medicinal chemistry, neurobiology, biochemistry, physiology, and other related biological sciences to problems of pharmacological interest. These predoctoral programs also are available to students in disciplines other than pharmacology. Regardless of their departmental affiliation, all trainees receive a thorough foundation in pharmacological principles and their application to all aspects of biomedical research. In FY 1985, there were 38 institutional predoctoral training programs, supporting 285 trainees.

provided through various funding mechanisms. Institutional postdoctoral fellowship programs in clinical pharmacology and anesthesiology are designed to train physicians in the application of laboratory research principles to the study of these disciplines. In FY 1985, there were 9 clinical pharmacology training programs supporting 28 trainees and 5 anesthesiology training programs supporting 8 trainees. Individual postdoctoral fellowships also are available in these disciplines. Research career development award applications are accepted by the Program only from those investigators who have expressed a commitment to research careers in clinical pharmacology or anesthesiology. The PS Program currently supports two such investigators in anesthesiology and one in clinical pharmacology.

The PRAT Program is the only intramural activity of NIGMS. This unique Program provides opportunities for 22 highly qualified postdoctoral fellows to spend 2 years in the intramural laboratories of the National Institutes of Health and the Alcohol, Drug Abuse, and Mental Health Administration. There are currently 86 formally appointed preceptors whose interests include pharmacokinetics, hormone-receptor interactions, cyclic nucleotide biochemistry, molecular toxicology, and virtually all aspects of the neurosciences. In addition to the extramural support for clinical pharmacology, the PRAT Program provides an individualized curriculum for interested and qualified physicians to pursue research training in clinical pharmacology at the National Institutes of Health.

PHARMACOLOGICAL SCIENCES PROGRAM

Program Area Summary
Fiscal Year 1985

	Trainee Positions	Awards	Dollars (In Thousands)
RESEARCH			
Research Grants			
Centers		4	2,128
Program Projects		8	3,076
Research Projects		431	52,693
Total		443	57,897
Career Program			
Career Awards		1	32
Career Development Awards		2	91
Total		3	123
Conference Grants		2	3
TOTAL RESEARCH		451	58,146
RESEARCH TRAINING			
National Research Service Award			
Institutional Fellowship (T32)			
o Predoctoral - Pharmacological Sciences	273	33	3,679
o Postdoctoral - Clinical Pharmacology	28	9	815
Anesthesiology	8	3	175
Total Institutional Training	309	45	4,669
Individual Fellowships (F32)			
o Postdoctoral	52	52	1,030
TOTAL FELLOWSHIPS	361	97	5,699
Intramural NIH			
Pharmacology Research Associates	22		616
TOTAL RESEARCH TRAINING	383		6,315
TOTAL IN PROGRAM AREA	383	548	64,461

RESEARCH HIGHLIGHTS

Receptors

"Regulation of Cyclic Nucleotide Metabolism"
R01 GM 34497-05 (Gilman, A.), University of Texas Health Sciences Center, Dallas
"Molecular Basis of Receptor-Cyclase Coupling"
R01 GM 27800-05 (Bourne, H.), University of California, San Francisco

Members of a family of membrane-bound guanine nucleotide-binding regulatory proteins (G proteins) serve as information transducers; they act as coupling factors to transmit information from receptors for a variety of regulatory compounds, such as hormones, neurotransmitters, or certain drugs, to effector proteins. G proteins control the activity of hormone-sensitive adenylate cyclase in essentially all cells, the activity of a photon-activated cyclic GMP-specific phosphodiesterase in the retinal rod outer segment, and, presumably, the activity of other proteins as well. Because of their key role, they have been the focus of interest for several investigators.

All G proteins that have been characterized to date consist of three components, α, β, and γ. Dr. Alfred Gilman determined the amino acid sequence of a region that appears to be common to the α subunits of all G proteins, as well as a portion of the *ras* oncogene product. He then used this amino acid sequence to produce an oligonucleotide probe (a 36mer with minimal redundancy) and, in turn, used this to screen a bovine brain cDNA library. The only clone that hybridized with this probe has a cDNA insert of approximately 1700 base pairs. Analysis of protein and DNA sequences indicates that the clone codes for a G protein that is distinct from both $G_{o\alpha}$, the most common G protein in bovine brain, and the α subunit of transducin. A peptide was synthesized according to the deduced amino acid sequence of the cDNA clone and was used to produce antibodies. These antibodies reacted specifically with $G_{s\alpha}$, the G protein that stimulates adenylate cyclase activity. In addition, RNA that hybridizes with probes made from the clone is detectable in wild type murine S49 cells by Northern analysis. However, mutant (cyc^-) S49 cells, which are essentially devoid of $G_{s\alpha}$ activity, lack this mRNA. Thus, the cDNA clone probably corresponds to $G_{s\alpha}$. This work will greatly facilitate studies of the synthesis and action of Gs under normal and pathological conditions.

Dr. Henry Bourne has focused his attention on transducin, a guanine nucleotide binding protein of retinal rod cells that mediates the activation of a cyclic GMP phosphodiesterase in response to photoexcitation of rhodopsin. This signal transducing protein (component molecular weights: α = 39,000; β = 35,000; and γ = 8,000) exhibits striking structural and functional homologies with the stimulatory (G_s) and inhibitory (G_i) coupling proteins of hormone sensitive adenylate cyclase. Dr. Bourne's laboratory recently isolated and sequenced a cDNA from bovine retina that encodes the α subunit of transducin. The cDNA was isolated with an antibody probe from a bovine retinal cDNA library in *lambda* gt11. The 2200-base long cDNA hybridizes to a single mRNA species in extracts of bovine retina but not of other tissues. The peptide sequence predicted by the cDNA includes the amino acids ADP-ribosylated by cholera and pertussis toxins. It also includes four segments, 11 to 19 residues long, that exhibit significant homology to 4 regions found in several other GTP-binding proteins, including the elongation factors of ribosomal protein synthesis in bacteria, EF-G and EF-Tu,

and the ras proto-oncogene products of man and yeast. Because previous genetic and biochemical evidence indicates that each of these four regions participates in binding or hydrolysis of GTP in the other proteins, the four homologous segments in the α subunit of transducin presumably form part of the GTP binding site. The amino acid sequence deduced in this study, in combination with previous functional studies of tryptic fragments, make it possible to predict which portions of the polypeptide interact with other molecules involved in retinal phototransduction, including the $\beta\gamma$ component of transducin, rhodopsin, and phosphodiesterase.

Studies from other laboratories have shown homology between the carboxyl terminus of transducin's γ subunit and the carboxyl terminus of the ras proto-oncogene product, p21, that is comparable in degree to the homologies between amino terminal sequences of the GTP-binding domain of p21 and amino terminal sequences of the α subunit of transducin. Thus, both the ras and G/transducin protein families may derive from a common evolutionary precursor.

"Adenylate Cyclase and the Plasma Membrane"
R01 GM 34571-09 (Neer, E.), Brigham and Women's Hospital, Boston
"Effect of Membrane Structure on Adenylate Cyclase"
R01 GM 30355-04 (Ross, E.), University of Texas Health Sciences Center, Dallas
"Kinetic and Chemical Properties of Beta Adrenergic Receptors"
R01 GM 33710-01 (Levitski, A.), Hebrew University of Jerusalem

Several research groups are isolating the components of the adenylate cyclase regulatory system, are characterizing them, and then are reconstituting the components back into functional, purified complexes. There is substantial evidence that the active component of the guanine nucleotide regulatory proteins can be ADP-ribosylated by a toxin from Bordetella pertussis. In her studies of the α components of inhibitory guanine nucleotide regulatory proteins in brain, Dr. Eva Neer has identified a second substrate (α_{39}) for ADP-ribosylation, in addition to the 41,000 molecular weight protein (α_{41}) found in many tissues. She has purified both pertussis toxin substrates from brain in free form and also as heterotrimers associated with a 36,000 molecular weight β protein and a 6,000 molecular weight γ protein. ADP-ribosylation of the two pertussis toxin substrates is greatly enhanced by adding the $\beta.\gamma$ component. This makes it possible to measure $\beta.\gamma$ activity, via its interaction with the α proteins.

Dr. Neer has developed polyclonal antibodies to α_{39} and to β. Polyclonal antibodies to α_{39} crossreact only weakly with α_{41}, indicating some homology between the two proteins but making it unlikely that one is derived from the other. Quantitative antibody measurements show α_{39} to be 0.5 percent, and β to be 0.7 percent, of total membrane proteins. Anti-β antibody recognizes the β subunit when it is associated with α_{39} or α_{41} and can immunoprecipitate both $\alpha.\beta.\gamma$ trimers. The antibodies crossreact with α_{39} and β proteins in many different species, but there always is more immunoreacting material in central nervous system tissues than in peripheral organs.

Dr. Neer has examined the conformational states of α_{41} and α_{39} (both ADP-ribosylated and unmodified), and $\beta.\gamma$ by limited proteolysis with trypsin. These studies show that the conformation induced by GTP differs from that induced by nonhydrolyzable guanine nucleotides, and both differ from that produced by ADP-ribosylation. The general similarity of the proteolytic products of α_{39} and

α_{41} to each other and to those reported for retinal transducin reinforce the idea that these proteins form a closely related family.

In independent projects, Drs. Elliott Ross and Alexander Levitski have purified β-adrenergic receptors and the activating GTP-binding regulatory protein (G_s) and have reconstituted them into unilamellar phospholipid vesicles in order to study the molecular basis by which their activity is regulated. Dr. Ross has found that mild reduction of disulfides on the receptor causes its activation as if it had bound an agonist. Receptors that have been reduced with dithiothreitol or 2-mercaptoethanol can activate G_s to about the same extent as can non-reduced receptors that are bound to isoproterenol, a β-adrenergic agonist. This is true whether the thiol-reduced receptors are unliganded or bound to an antagonist ligand. If the receptors are reduced and then allowed to bind an agonist, their regulatory effect is far greater than that of non-reduced agonist-liganded receptors. As maximal activity of the receptors requires both the presence of agonist and the reduction of disulfides, thiol-disulfide equilibria may be important in regulating the effects of β-adrenergic systems.

In his work with turkey erythrocyte β-adrenoceptors, Dr. Levitski found the β-adrenoreceptor to be tightly associated with a ganglioside. ^{125}I-Bromoacetyl-aminomethyl-cyanopindolol, a β-receptor affinity label, blocks the ligand binding site and labels the tightly associated gangliosides. However, receptor binding activity can be recovered after dissociation of the labelled ganglioside from the β-receptor protein by gel permeation chromatography in the presence of the detergent deoxycholate. Dr. Levitski has reconstituted the β1-adrenergic receptor and the GTP regulatory protein G_s into phospholipid vesicles and has shown that interaction between the receptor and G_s occurs via "collision coupling;" that is, the β-receptor catalytically activates as many G_s units as are accessible within the vesicle. Furthermore, the βγ subunits of G_s are essential for the guanyl nucleotide exchange at the α_s subunit of G_s.

"Receptors in a Smooth Muscle Cell Line"
R01 GM 30669-02 (Cornett, L.), University of Arkansas Medical Sciences

Much less is known at the molecular level about the structure and function of the α_1-adrenergic receptor than is known about the β-adrenergic system. This is due partly to the relatively low abundance of α_1-adrenergic receptors in most tissues and cell types.

During the past year, Dr. Lawrence Cornett and his collaborators have examined a smooth muscle cell line, designated DDT$_1$ MF-2, as a model for research on α_1-adrenergic receptors. These cells grow rapidly in suspension cultures to high densities and possess high numbers of functional α_1-adrenergic receptors (65,000 receptors/cell) but no detectable α_2-adrenergic receptors.

Dr. Cornett has labeled covalently the α_1-adrenergic receptor of these cells with two different affinity ligands. Incorporation of the affinity ligands into the receptor could be blocked by adrenergic agonists and antagonists in a manner consistent with α_1-adrenergic receptor interaction. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis under reducing conditions revealed a molecular weight for the receptor of 81,000 daltons. Binding of epinephrine or norepinephrine to the α_1-adrenergic receptor of the cultured cells causes release of calcium from an intracellular calcium pool, thereby transiently increasing cytosolic calcium

levels. This effect could be blocked by prazosin and phenoxybenzamine, which are α_1-adrenergic receptor antagonists. These findings form the basis for in-depth research on the structure, function, and organization of the α_1-adrenergic receptor.

"Mutants of an Apparent Acetylcholine Receptor"
R01 GM 31659-03 (Lewis, J.), University of Missouri, Columbia

The nematode Caenorhabditis elegans seems to have an acetylcholine receptor that appears to be required for proper motor behavior but to be otherwise dispensable. The receptor can be assayed in vitro by the binding of tritiated meta-amino-levamisole, a nicotine-like drug. Mutants for the receptor can be selected by their resistance to the toxic agonistic effects of the parent drug, levamisole. Seven genes have been identified which, in the mutated state, lead to significantly altered receptor binding properties. In order to understand the role of each of these genes, Dr. James Lewis has generated monoclonal antibodies against the nematode receptor, using the tiny quantities of partially purified receptor that he has been able to obtain. To date, he has identified three monoclonal antibodies. One monoclonal antibody detects a receptor peptide of approximately 43,000 daltons molecular weight. A second peptide of approximately 38,000 molecular weight also appears to be part of the receptor structure, as this peptide is removed from solution by immunoprecipitation of the native receptor.

The recent discovery of a nematode transposable genetic element makes cloning the nematode receptor genes possible. By expressing the cloned genes in bacteria, it should be possible to generate large amounts of material for a variety of studies. Dr. Lewis has isolated 25 spontaneous receptor mutants in the Bergerac strain of C. elegans, where a major fraction of spontaneous mutants are caused by mutational insertion of the transposon TC1. Spontaneous mutants have been found so far for five of the seven receptor genes. The same transposon-associated Hind III restriction fragment has been found in all four unc-38 gene mutants examined but not in any of the five unmutated control strains. Dr. Lewis currently is cloning the unc-38 gene and is identifying and cloning restriction fragments associated with the TC1-caused mutation of other receptor genes.

"Biogenesis of Acetylcholine Receptor"
R01 GM 32629-03 (Hawrot, E.), Yale University

Snake curaremimetic neurotoxins are known to bind to the nicotinic acetylcholine receptor and to block its function by preventing the binding of the natural neurotransmitter, acetylcholine. Dr. Edward Hawrot has been investigating the neurotoxin binding site of the alpha subunit of the acetylcholine receptor. Neurotoxins are able to bind to this subunit even after it has been separated from the three other subunits that make up the oligomeric active receptor complex. In fact, some proteolytic fragments of the alpha subunit still bind neurotoxin even though they may be only one-fifth the size of the intact alpha subunit. The neurotoxin binding site has been localized to a 100 amino acid long region of the alpha subunit.

Dr. Hawrot extended this study further by synthesizing a peptide containing only the 32 amino acid sequence postulated to comprise the neurotoxin binding site. He has found that this synthetic peptide binds α-bungarotoxin with the same affinity and specificity as does the isolated, intact alpha subunit. This research should lead to a definition of the exact molecular interactions that

occur between amino acid residues of the binding site and the bound antagonist and should aid understanding of how the binding of acetylcholine leads to activation of the receptor's channel function.

Hormones

"Isolation of Compounds Promoting Cell Division in Plants"
R01 GM 33585-02 (Lynn, D.), The University of Chicago

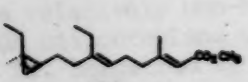
Crown gall tumor disease is caused by the soil bacterium Agrobacterium tumefaciens, through the transfer of a DNA segment (T-DNA) from one of its plasmids (Ti-plasmid) into the nuclear genome of the infected plant cell. This genetically transformed and bacterially engineered cell is capable of producing several metabolic products, such as nopolines and octopines, that generally are not found in normal cells, and is capable of autonomous proliferation in the absence of phytohormones.

Dr. David Lynn has purified six factors from transformed Vinca cells that promote cell division in normal plant tissue. These compounds have similar biological activity, appear to be structurally related, and exist in the tumor tissue in the same relative concentrations through several subculturing cycles. Common structural features include a heterocyclic base containing four nitrogen atoms, a β-linked glucose residue, two N-methyl groups, an allylic alcohol, and another undefined hydroxylated carbon chain. These compounds seem to differ structurally from any previously characterized plant product.

Tobacco pith tissue cultured in the presence of plant hormones produces all six of these growth factors in the same relative ratios as the transformed tumor cells. In contrast, the spectrum of these materials in uncultured tobacco plant material is very different. Therefore, the oncogenetic nature of the transformed DNA seems to be related to overproduction of normal cell division-promoting factors—the same factors whose production normally is controlled by plant hormones. This finding is consistent with the initial epigenetic mechanism for tumorigenesis in crown gall tumor proposed by Braun many years ago and agrees with recent findings in several laboratories regarding homologies between mammalian oncogene products and peptide growth factors. The crown gall tumor seems to mimic animal tumors in general and may provide a simplified system in which to study transformation from normal to malignant cell growth. In addition, the structural definition of these factors may lead to an understanding both of plant cell growth and of the control points in the cell cycles in all eukaryotic systems.

"Photoaffinity Labels for Insect Juvenile Hormone Binding Proteins"
R01 GM 30899-02 (Prestwich, G.), State University of New York, Stonybrook

Dr. Glenn Prestwich has developed a new stereoselective total synthesis of chiral juvenile hormone I (JH I) that allows introduction of two tritium atoms in the final step. Both optical antipodes of the pivotal epoxy alcohol intermediate



JH I

have been prepared in 95 percent enantiomeric excess via the Sharpless epoxidation of a (Z)-allylic alcohol. Elaboration of the hydroxymethyl group to a vinyl group, followed by selective homogenous tritiation, affords optically active juvenile hormone I analogs at 58 Curies per nanomole. Dr. Prestwich has used the labeled enantiomers (10R,11S and 10S,11R) to study their binding to the hemolymph binding protein of Manduca sexta larvae, employing a dextran-coated charcoal assay. The natural (10R,11S) enantiomer had twice the relative binding affinity as did the (10S,11R) enantiomer. The availability of such high specific activity, optically pure hormones will contribute substantially to research on high-affinity receptors for juvenile hormones. Moreover, the chiral 12-hydroxy-(10R,11S)-epoxy intermediate allows modification of juvenile hormone for solid-phase biochemical and radioimmunochemical studies without altering either the biologically important carbomethoxy or epoxy recognition sites.

The new radioactive and radioinert hormones have been used to monitor juvenile hormone binding activity during the purification of these proteins from the hemolymph of late instar larvae of Heliothis virescens (Noctuidae), Lymantria dispar (Lymantriidae), Manduca sexta (Sphingidae), and Trichoplusia ni (Noctuidae). These studies eventually could lead to the development of more selective and effective insecticides.

"ACTH and Related Peptides"
R01 GM 02907-34 (Li, C.), University of California, San Francisco

β-Endorphin-(1-27) is a naturally occurring peptide found in pig, rat, and horse pituitary glands and also in brain. This peptide consists of the first 27 amino acid residues of β-endorphin, which itself is 31 residues long. Dr. Choh Li recently showed that human β-endorphin-(1-27) inhibits the analgesia that normally is produced by either human β-endorphin or by etorphine, which is a potent analog of morphine. Because of its properties, Dr. Li named the shorter peptide β-endorphin inhibiting peptide. Its inhibition of analgesia is dose-related and effective during the entire time course of analgesia that normally would be induced by these highly potent agonists. Quantitative analyses of the inhibition by Schild plots are consistent with a competitive antagonism. Human β-endorphin inhibiting peptide is four to five times more effective than naloxone for inhibiting etorphine-induced analgesia.

These results with the endorphins are analogous to what Dr. Li found earlier for the corticotropins. Human β-corticotropin (7-38) is a naturally occurring segment consisting of a portion of the amino acid sequence of corticotropin, which itself is 39 residues long. This peptide was isolated from human pituitary glands and shown in 1978 to inhibit corticotropin action. It therefore was named corticotropin-inhibiting peptide. These observations give rise to the concept of a peptide segment within the amino acid sequence of a hormone that inhibits the action of the hormone itself.

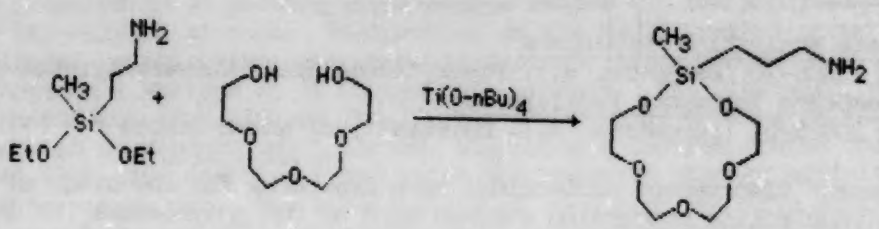
Nucleotides

"Phosphate Activation Mechanisms"
R01 GM 13306-20 (Benkovic, S.), Pennsylvania State University, University Park
"Cell Membrane Transport Facilitators"
R01 GM 30805-01 (Bergstrom, D.), University of North Dakota

Most present fluorescent nucleotides have drawbacks for the study of the important polynucleotide processing enzymes such as DNA polymerase, DNA ligase and various restriction enzymes, since they distort proper-base pairing, prevent chain elongation, or have severely quenched fluorescent properties when in a polynucleotide such as DNA. Dr. Stephen Benkovic recently developed two new fluorescent deoxyuridine triphosphates that may have a variety of applications. These compounds, which contain 1-aminonaphthalene-5-sulfonic acid moieties, are substrates for DNA polymerase I and can be incorporated into duplex DNA. The binding of protein to DNA enhances their fluorescence. They provide a convenient continuous assay of the elongation of the DNA by polymerase. Furthermore, when used in conjunction with protein labelled with an acceptor fluorescent probe, fluorescently labelled DNA or the original nucleoside triphosphate can serve as an energy transfer donor, thereby furnishing estimates of the distance between the fluorescent label on the bound DNA or nucleoside triphosphate and that on the protein. Thus, these fluorescent compounds should greatly facilitate structure/function studies of important interactions involving nucleotides and polynucleotides.

Dr. Donald Bergstrom has prepared and tested two fluorescent nucleosides, 3-β-D-ribofuranosyl-2,7-dioxopyrido (2,3-α) pyrimidine and ethenoadenosine, as reagents for detecting nucleoside transport, in order to monitor and to facilitate the delivery of nucleotide-derived antiviral and antitumor agents into target cells. His approach was based on the predicted ability of these nucleosides to transfer their fluorescence emission to a second species, which is impermeable to a phospholipid bilayer. He used detergent dialysis to construct unilamellar liposomes containing a non-transportable fluorescent chromophore or a fluorescence quencher, such as relatively low concentrations of iodide ion or cobalt II chloride, with an excitation band that overlaps the emission band of the fluorescent nucleoside. When the nucleoside was introduced into the solution outside the liposome, Dr. Bergstrom found that it fluoresced normally; however, when the nucleoside is transported into the interior, its fluorescence is quenched.

The pharmacological effectiveness of antiviral agents such as arabinosyladenosine is substantially diminished because the compounds are rapidly metabolized *in vivo*. Their corresponding nucleotides (nucleoside 5'-phosphates) are not therapeutically useful either because, almost without exception, the obligatory bioactive intermediate is too hydrophilic to be able to pass through lipid bilayers. Nucleotide derivatives which contain a cyclic polyether (crown ether) linked through the phosphate moiety are soluble both in aqueous solution and in relatively lipophilic solvents like chloroform, but simple crown ethers are cytotoxic, apparently because of their hydrolytic stability and tendency to partition into the lipophilic phase. Researchers at Petrarch Chemical Company recently described a new class of crown ethers that seem to be relatively non-toxic, probably because they hydrolyze slowly *in vivo* to innocuous oligoethylene glycols. Using the results of the Petrarch group as a guide, Dr. Bergstrom synthesized silacrown ethers, such as 1, by the reaction shown below.



1, bp 136°/0.025mm

Because of the amine functional group, 1 and related analogs should be useful not only for the derivatization of nucleotides, but also as a selective cation binding ligand that could easily be attached to the carboxyl group of many different classes of compounds or could be immobilized on a solid support.

"Bioorganic Chemistry of Nucleic Acid Alkylphosphonates"
R01 GM 31927-02 (Miller, P.), Johns Hopkins University

Dr. Paul Miller of Johns Hopkins University is seeking to develop oligonucleotide analogs that can control gene expression at the level of DNA transcription or at the level of mRNA translation or processing. Toward this goal, he has used solid phase techniques to synthesize oligodeoxyribonucleoside methylphosphonates of defined base sequence that are specifically designed to be taken up intact by mammalian cells in culture and to form stable, hydrogen-bonded complexes with complementary target DNA or RNA sequences. Oligomers up to 15 nucleoside units long have been synthesized and characterized by procedures developed by Dr. Miller. He has found that oligomers complementary to the splice junction sequences of SV40 precursor mRNA selectively inhibit virus protein synthesis in virus-infected cells. Herpes simplex virus type 1 DNA synthesis, protein synthesis, and virus growth are selectively inhibited in virus-infected Vero cells by an oligomer which is complementary to the exon/intron junction of an immediate early mRNA.

Dr. Miller recently designed a new type of methylphosphonate oligomer that forms covalent crosslinks with its target nucleic acid. The oligomer contains a derivative of psoralen attached to its 5'terminal nucleotide residue that, when irradiated with 365 nm light, forms adducts with cytosine, uridine or thymine residues of the complementary nucleic acid strand. In preliminary experiments utilizing very low concentrations of psoralen-derivatized methylphosphonate oligomer complementary to β globin mRNA, the compound formed covalent complexes between the complementary oligomer and mRNA after irradiation, resulting in a 75 percent inhibition of translation of the mRNA. As the specificity of the oligodeoxyribonucleoside methylphosphonates resides in their ability to bind to complementary nucleic acid sequences, it should be possible to use nucleic acid sequence information to design novel derivatives of methylphosphonate oligomers which can be used to study gene expression and the function of specific proteins in normal, transformed or virus-infected cells.

"Metal Complexes of Nucleotides and Coenzymes"
R01 GM 26390-06 (Clarke, M.), Boston College

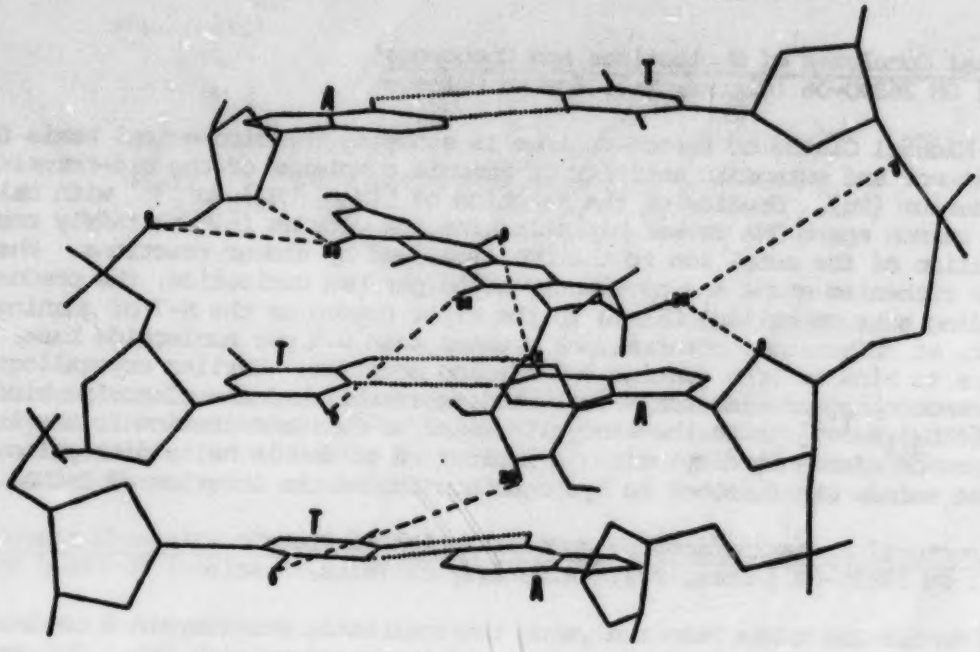
Dr. Michael Clarke of Boston College is studying the biochemical basis for the antitumor and mutagenic activity of ammonia complexes of the mid-transition metal, ruthenium (Ru). Studies of the reaction of $[(H_2O)(NH_3)_5Ru^{II}]^{2+}$ with calf thymus and salmon sperm DNA reveal biphasic kinetics with an initial fairly rapid coordination of the metal ion to the DNA, followed by slower reactions. When up to 0.26 ruthenium atoms are covalently bound per DNA nucleotide, the predominant binding site on helical DNA is in the major groove at the N-7 of guanine. However, at ruthenium concentrations greater than 0.5 per nucleotide base, ruthenium seems to bind to both guanine and adenine moieties. Earlier crystallographic, spectroscopic and electrochemical studies established the adenosine binding site of $[(NH_3)_5Ru^{III}]$ to be the exocyclic amine, N-6. Coordination to the exocyclic amines of adenine and cytosine is indicative of double helix disruption, since these amines are involved in hydrogen bonding on the interior of B-DNA.

"Structural Investigations of Mode of Action of Drugs"
R01 GM 32690-03 (Arora, S.), University of Texas, Austin

Dr. Satish Arora has been analyzing the antibiotic steffimycin B to determine its structure, absolute stereochemistry, and interactions with DNA. Its crystals belong to the orthorhombic space group $P2_12_12_1$ with cell dimensions of a = 8.253, b = 8.198, and c = 40.850Å. The structure was solved by direct methods and refined to an R of 0.066.

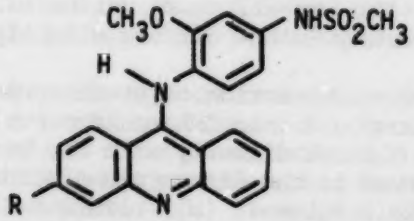
The configuration in Ring A is 7R, 8S, 9S. Since the configuration of the sugar was determined earlier, these latest results establish the complete absolute stereochemistry of steffimycin B. The conformation of ring A is half chair, similar to that observed in daunomycin, carminomycin and nogalamycin. The hydroxyl O(9), which is very important for biological activity, is equatorial, while C(13) is axial. The overall conformation of steffimycin B resembles that of daunomycin and carminomycin but differs slightly from nogalamycin.

A possible model for the interaction of steffimycins with DNA is shown below. Intercalation of the compound into DNA would occur first, followed by hydrogen bonding. Rings B and C stack directly with the base pairs, while rings A and D protrude just as observed in the daunomycin-d(GCTAGC) complex. The hydrogen bonding scheme probably involves: (a), bifurcated hydrogen bonds with O(4) as donor to oxygen atoms of backbone phosphate and sugar; (b), hydroxyl O(6) and carbonyl O(2) of thymine; (c), O(9) as bifurcated donor to oxygens of phosphate and sugar; (d), O(12) of the antibiotic as receptor from the amino group of adenine of the middle base pair; and (e), O(3') as donor to O(2) of the thymine of the bottom base pair. The hydrogen bonding in this proposed complex is weaker than that observed in the daunomycin-DNA complex, in accord with the weaker biological activity of steffimycins.



"Mode of Action of Mutagenic Drugs"
R01 GM 29175-03 (Byrn, S.), Purdue University

Acridines of the AMSA class are of substantial interest because AMSA is undergoing Phase II two clinical trials and has promising antitumor activity. For this reason, it is important to understand the mechanism of mutagenesis of this compound.



AMSA (R = H)
azido AMSA (R = CH_3)

Acridines of the AMSA class are mutagenic in both Ames strain TA1537 and in the L5178Y mammalian mutagenesis assay, but they are not mutagenic in the V79 mammalian mutagenesis assay. However, the azido AMSA derivative is mutagenic in all three of these assays when exposed to ultraviolet. This suggests that covalent binding, which occurs upon exposure of the azido AMSA to light, is related to mutagenesis in the V79 assay but not for either the Ames TA1537 assay or the L5187Y assay.

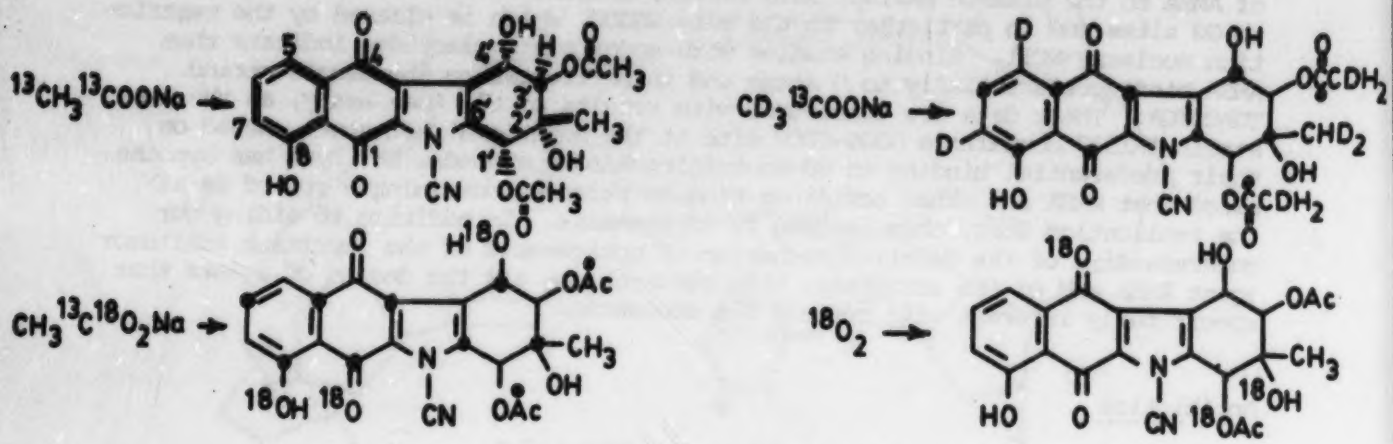
Dr. Stephen Byrn has used restriction endonucleases to investigate the binding of AMSA to the plasmid pAT153. His studies indicate that AMSA binds most strongly to GG sites and in particular to the site GGGCC, which is cleaved by the restriction nuclease NCil. Binding studies with model octanucleotides indicate that AMSA binds preferentially to G sites and in particular to the single strand TGAGGTGA. These data are consistent with results of the Ames assay, as the Ames strain TA1537 contains a GGGG-CCCC site at the mutational hot spot. Based on their preferential binding to GG-containing single strands, Dr. Byrn has hypothesized that AMSA and other acridines bind to strands containing a run of Gs at the replication fork, thus leading to mutagenesis. In addition to aiding our understanding of the detailed mechanism of mutagenesis of the important antitumor agent AMSA and of the acridines, this research may aid the design of agents that specifically interact with certain DNA sequences.

Antibiotics

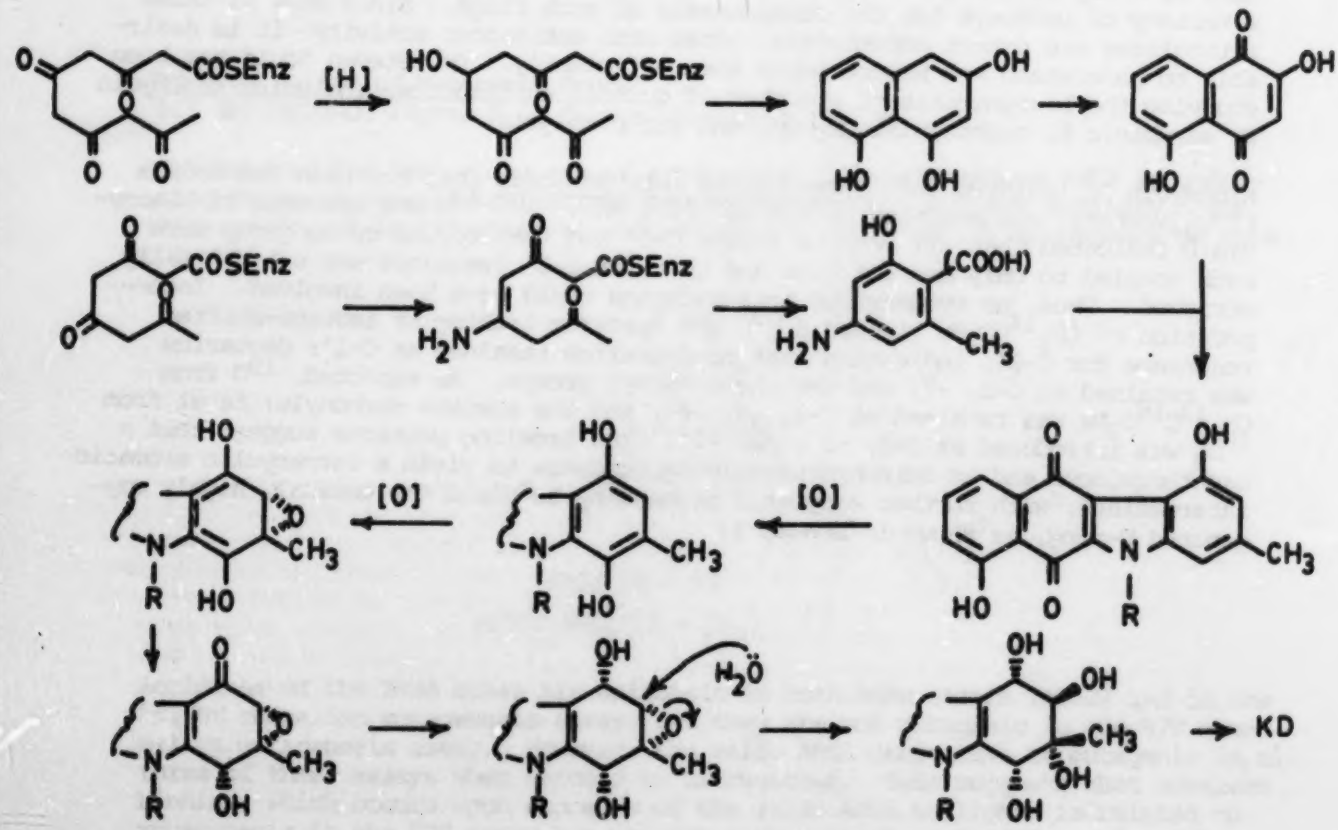
"Mutasynthetic and Bioorganic Studies of Antibiotics"
R01 GM 31715-03 (Gould, S.), Oregon State University

Microorganisms are particularly adept at making metabolites containing a quinone ring as a key functional unit. Studies from numerous laboratories have revealed a variety of pathways for the biosynthesis of such rings. Since many of these metabolites are potent antibiotics, often with anticancer activity, it is desirable to understand how Nature makes them efficiently. Dr. Steven Gould has been studying the biosynthesis of a number of quinoid antibiotics, including kinamycin D, sarubicin A, naphthyridinomycin, and streptonigrin.

Kinamycin D, isolated from Streptomyces murayamaensis that had been fed sodium [$^{13}C_2$]acetate, was analyzed by NMR at 100.6 Mhz. The ^{13}C NMR spectrum of kinamycin D indicated that all carbons except C-3' and that of the cyano group were each coupled to only one neighbor and that the C-3' resonance was substantially enriched. Thus, no symmetrical intermediates could have been involved. Incorporation of $CD_3{}^{13}CO_2Na$ yielded a ^{13}C NMR spectrum lacking an isotope-shifted resonance for C-6', indicating that no deuterium remained at C-1'; deuterium was retained at C-5, -7, and the three methyl groups. As expected, ^{18}O from $CH_3{}^{13}C^{18}O_2Na$ was retained at C-1, -8, -4', and the acetate carbonyls; label from $^{18}O_2$ was introduced at C-4, -1', and -2'. The labeling patterns suggest that a naphthoquinone and an aminohydroxytoluene condense to yield a tetracyclic aromatic intermediate, with further enzymatic processing to yield the unusual, highly oxygenated D-ring, as shown in Scheme 1.

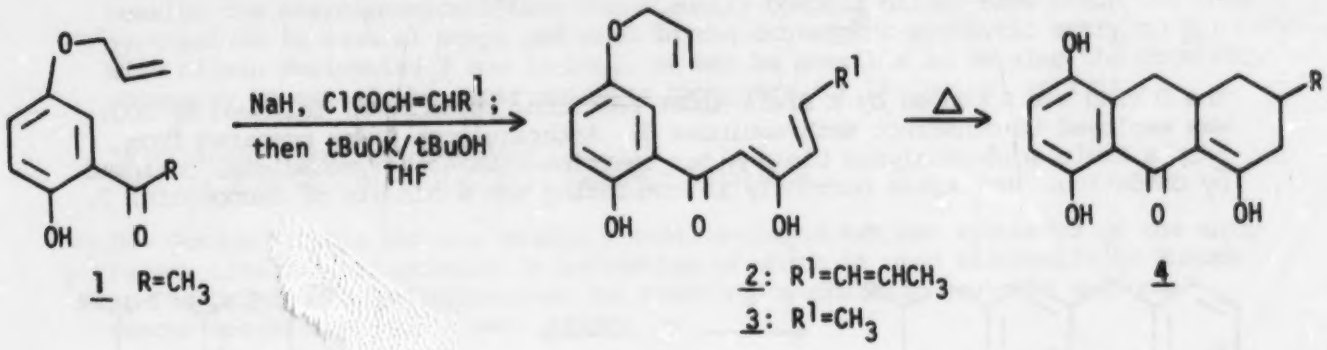

$^{13}CH_3{}^{13}COONa$
$CD_3{}^{13}COONa$
$CH_3{}^{13}C^{18}O_2Na$
$^{18}O_2$

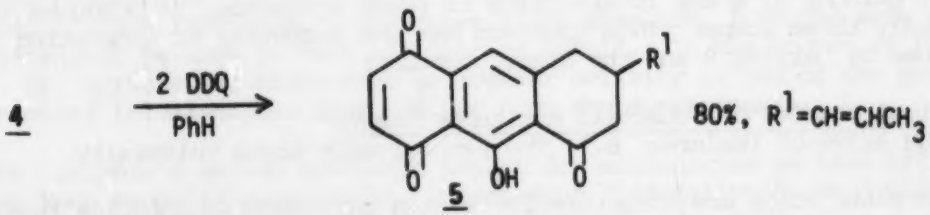
SCHEME 1


COSEnz
[H]
[O]
[O]
$H_2\ddot{O}$
KD

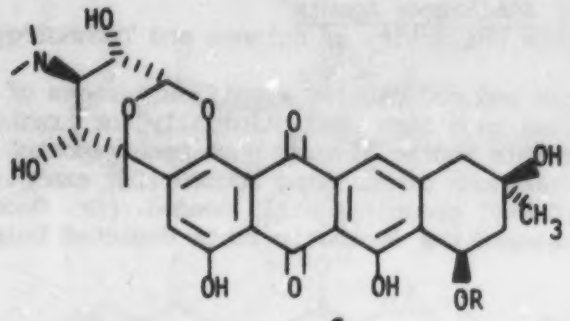
"Synthesis of Aromatic Antibiotics and Anticancer Agents"
R01 GM 34342-01 (Kraus, G.), Iowa State University of Science and Technology

The anthracyclines constitute a diverse and medicinally significant class of natural products. Members of this class have been used clinically for treating cancer. While several clever and workable routes already have been reported, a flexible synthesis which avoids the drawbacks of existing routes (for example, solvolytic introduction of the C-7 hydroxyl group) is still needed. Dr. George Kraus and his research group have developed the synthetic route depicted below.

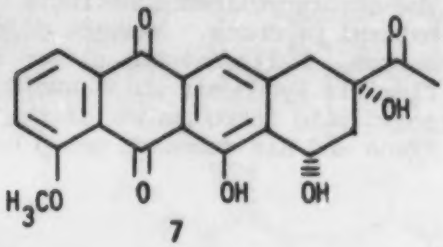




The central strategy involves coupling a regiospecific Claisen rearrangement with an intramolecular Diels-Alder reaction. This sequence, illustrated in the transformation of 3 into 4, proceeds in 60-84 percent isolated yield by simply heating a benzene solution of 2 or 3 at 210°C for 8 hours. Aromatization of the central ring in 4 then can be accomplished with two equivalents of the oxidant DDQ in benzene at ambient temperature. Quinone 5 contains the ABC ring system of the important 11-deoxyanthracyclines. Two members of this subclass of anthracyclines, the nogarols, 6, and 11-deoxy daunomycin, 7, are shown below.

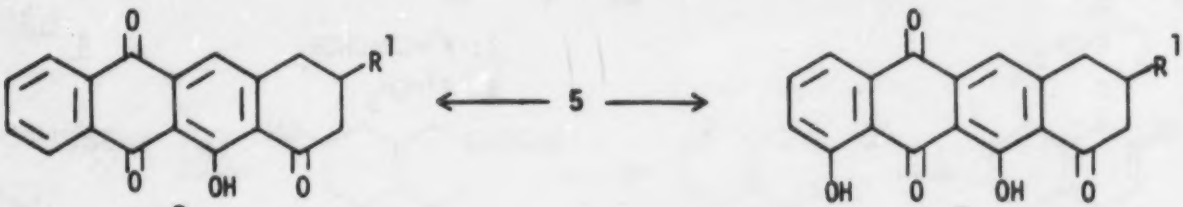


6



7

The D ring was appended by a Diels-Alder reaction. Butadiene, followed by DDQ, was employed to construct anthraquinone 8. Anthraquinone 9 was prepared from 5 by a Lewis acid-catalyzed Diels-Alder reaction with acetoxybutadiene, followed by oxidation. Dr. Kraus currently is completing the synthesis of daunomycin, 7.

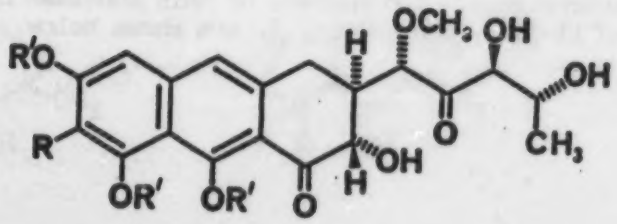


8 ⟵ 5 ⟶ 9

Hence, quinone 5, a key intermediate in these syntheses, is produced from compound 1 in only three steps. This approach has the potential of generating many anthracyclines by varying R and the diene component.

"Chemical Studies on Potential Antitumor Agents"
R01 GM 32299-07 (Weinreb, S.), The Pennsylvania State University

The aureolic acids are structurally related antitumor antibiotics that have been isolated from several species of Streptomyces. The principal members of this group are the olivomycins, the chromomycins, and mithramycin. The olivomycins were discovered in the USSR, the chromomycins in Japan and mithramycin in the USA. On acid hydrolysis, the olivomycins give di- and tri-saccharides and the aglycone olivin, 1. Hydrolysis of the chromomycins or mithramycin affords various sugars and the aglycone chromomycinone, 2.



1 R′=R=H

2 R′=H, R=CH₃

3 R′=CH₃, R=H

The aureolic acids currently are used clinically in cancer chemotherapy in their countries of origin; mithramycin has been approved for use in testicular cancer in the USA. However, all of the aureolic acids, like many other antitumor compounds, show toxic effects and side reactions.

Dr. Steven Weinreb is attempting to synthesize new compounds with related structures in the hope of producing better therapeutic agents. He and his coworkers recently completed the first total synthesis of one of the aureolic acid aglycones, tri-O-methylolivin 3. The synthetic approach utilizes a novel condensation reaction of an orsellinate ester and a β-methoxycyclohexenone that was specifically designed to create the tricyclic ring system of 3. A β-methoxycyclohexenone bearing the stereochemically and functionally complex olivin side chain was thus synthesized in several steps and used in the convergent synthetic entry to 3. With olivin derivative 3 now in hand, it may be possible to develop the chemistry necessary to attach the sugar moieties necessary for biological activity.

"Anticancer Alkaloid Synthesis"
R01 GM 30761-03 (Gribble, G.), Dartmouth College

Dr. Gordon Gribble and his students have developed two new syntheses of the anticancer alkaloid ellipticine, a derivative of which is used clinically in Europe and is proving to be efficacious for treating myeoblastic leukemia and bone cancer metastases from breast cancer.

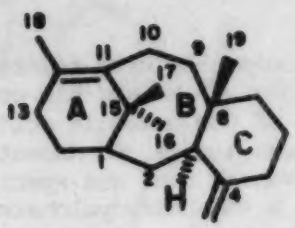
In one of the synthetic procedures, gram quantities of ellipticine can be produced from the common chemical indole in a matter of days in 55 percent overall yield, using six relatively simple chemical steps. This procedure has the advantage that the same chemical intermediate can be used to prepare an array of ellipticine derivatives for anticancer screening. For example, Dr. Gribble has synthesized 17-oxoellipticine, a Strychnos plant alkaloid, from the same chemical intermediate that he uses to make ellipticine itself. In the course of this research, Dr. Gribble has discovered anticancer activity in two of the pre-ellipticine chemical intermediates that had not been found previously.

So far, Dr. Gribble's second synthetic method for ellipticine is less efficient, but it has been especially valuable for preparing "bis-ellipticines," which may evolve into a new and more powerful class of anticancer drugs.

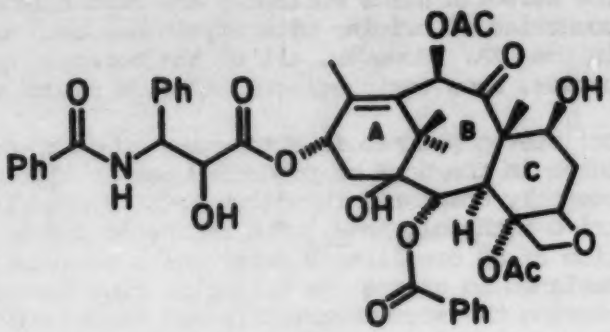
As the successful use of chemotherapy against solid tumors has been difficult, it is important that the ellipticine class of drugs—shown to be effective on solid tumors—be thoroughly studied.

"Organic Synthesis"
R01 GM 33482-02 (Shea, K.), University of California, Irvine

The taxane diterpene skeleton 1 forms the basis of a pharmacologically interesting class of natural products Isolated from the species Taxus Linnaeus. Taxol, 2, is the cytotoxic and antileukemic constituent of Taxus brevifolia Nutt; it is an antimitotic agent that acts by promoting microtubule formation.



1

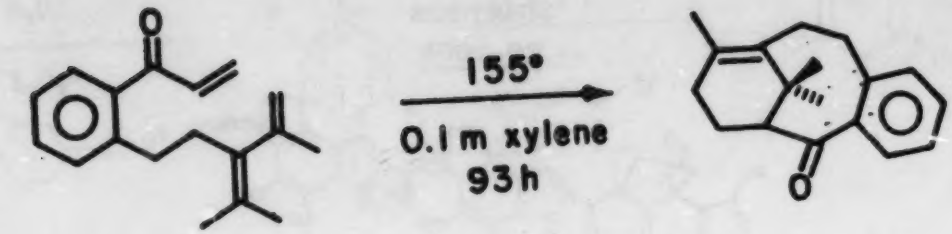


2

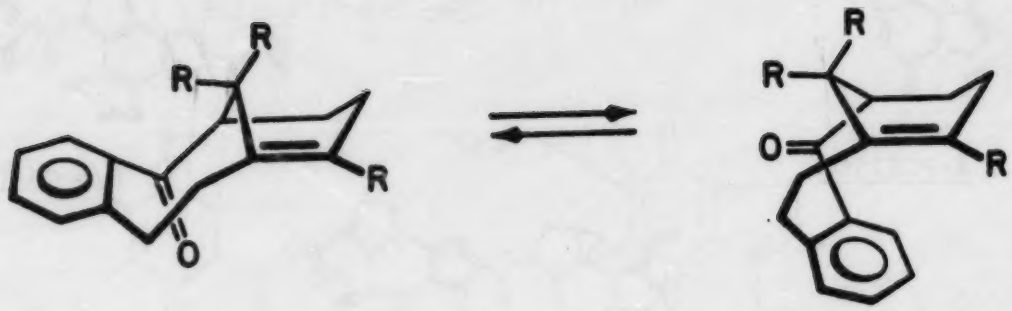
Dr. Kenneth Shea is developing general synthetic entries into the taxane family of natural products and has found an efficient entry into the tricyclo[9.3.1.$0^{3,8}$] pentadecane ring system, the key substructural skeleton of taxanes. His approach utilizes a type 2 intramolecular Diels-Alder cycloaddition to assemble the A and B rings in one step (see below). A very interesting aspect of this tricyclic ring

system is its conformational behavior. Through a complex sequence of torsions about carbon-carbon single bonds, the two conformational isomers, 3 and 4, are interconverted. In certain cases, the energy barrier separating these isomers is sufficiently high to permit their physical separation.

Dr. Shea currently is exploiting the conformational bias of the tricyclic ring system to direct the stereospecific entry of the C-8 methyl group for construction of the taxane skeleton. The methods being developed for synthesizing the taxane skeleton should lead to several derivatives for biological screening.



70%



exo 3 endo 4

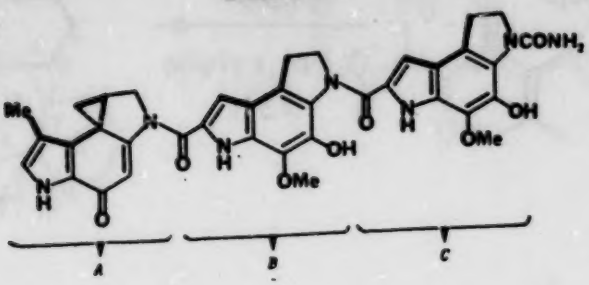
"Synthesis of the Antitumor Agent CC-1065"
R01 GM 32718-02 (Magnus, P.), Indiana University

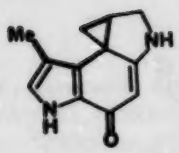
Several years ago, the potent antitumor agent CC-1065 was isolated and its structure determined by the Upjohn group. While this compound is 100 times more potent than actinomycin and vinblastine, it unfortunately produces acute liver toxicity. CC-1065, therefore, has been viewed as a promising lead compound, with the optimistic possibility that analogs will have its desirable effect in destroying cancerous cells but not its acute liver toxicity. Upjohn and Hurley found that CC-1065 binds the minor groove of DNA, and the incipient alkylating component, the spirocyclopropane, alkylates a basic nitrogen atom of adenine.

Dr. Philip Magnus has synthesized the A-portion of CC-1065 as the free diamine. The A portion does not bind DNA but it does inhibit protein synthesis. Indirect coupling of the A-portion to simple flat aromatic acids produces very promising analogs that possess cytotoxic properties but do not show liver toxicity. Synthesis of the separate B/C components is nearly complete and also should permit the construction of important analogs.

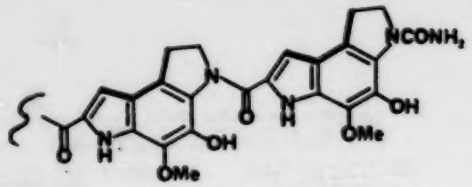
STRUCTURES

CC-1065

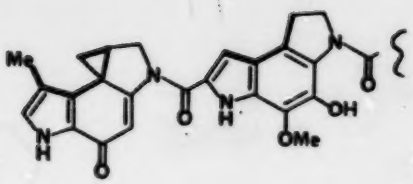




A-PORTION



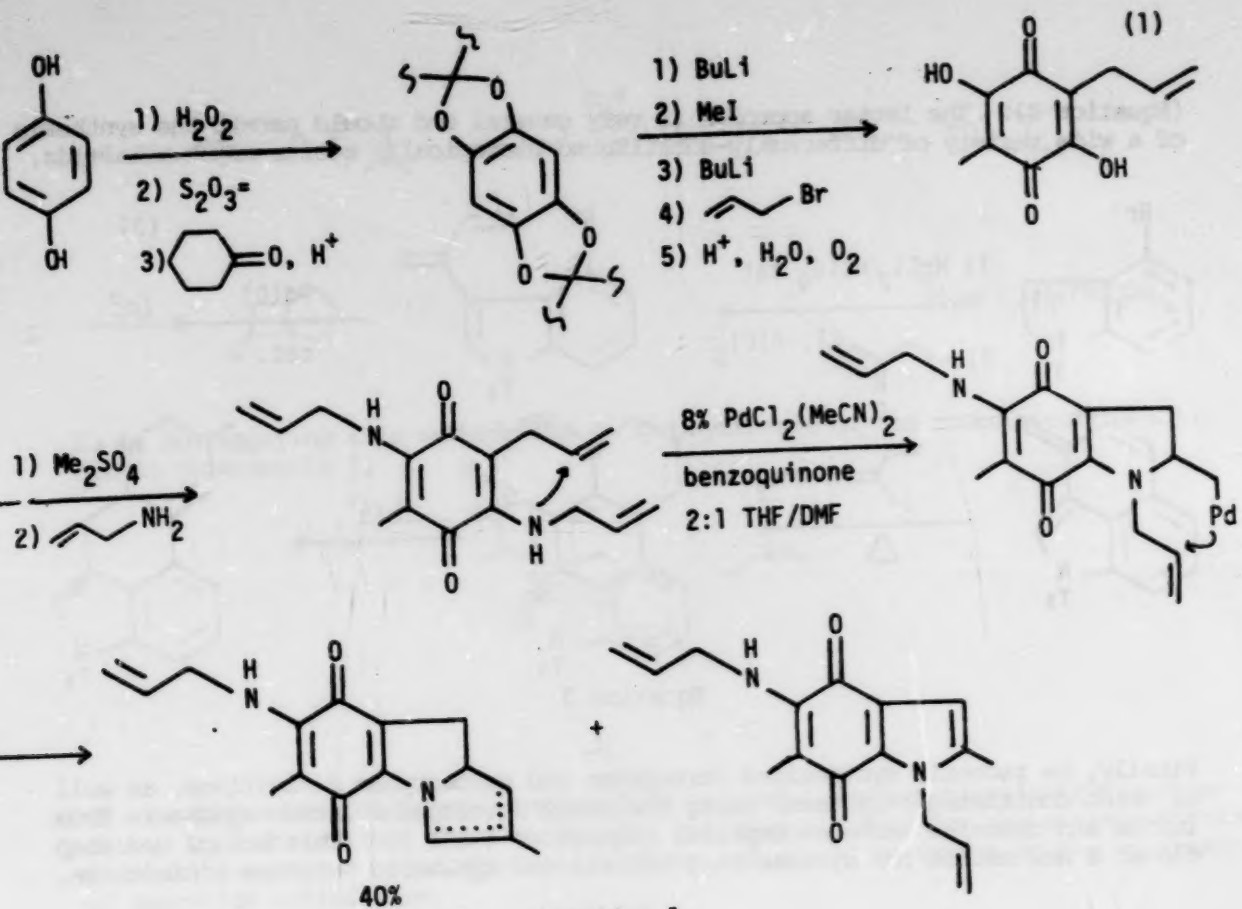
B/C-PORTION



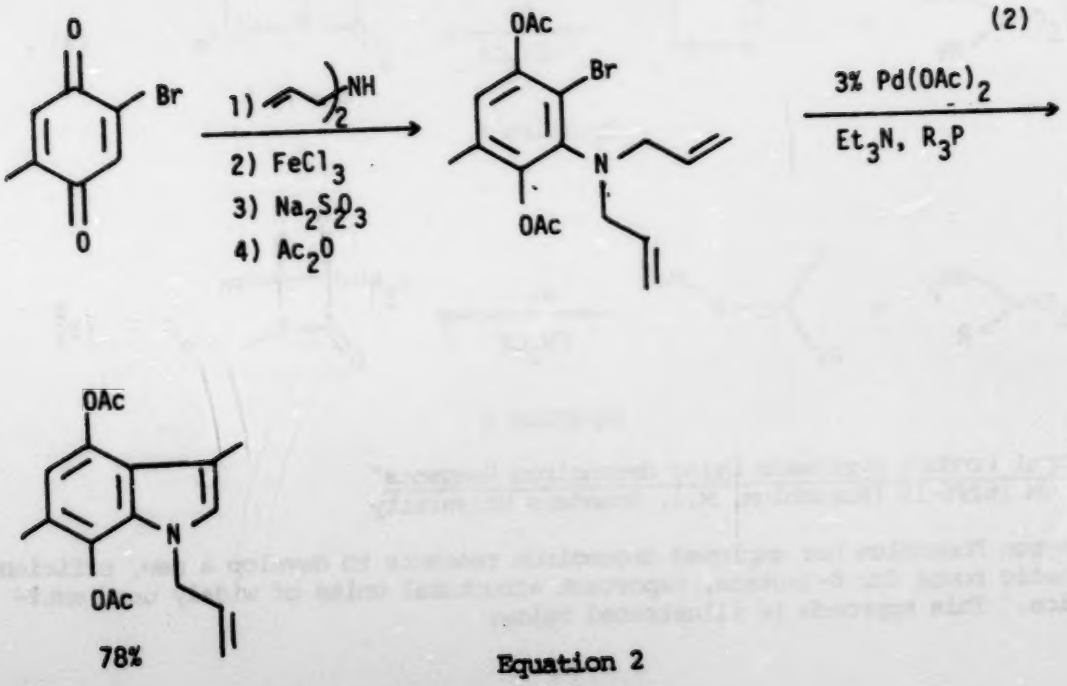
AB-PORTION

"Organometallic Synthesis of Bioactive Compounds"
R01 GM 26178-07 (Hegedus, L.), Colorado State University

Dr. Louis Hegedus is developing efficient synthetic approaches for three classes of biologically active compounds--the mitocenes, the ergots and the β-lactams. He has formulated a nine-step synthesis of the mitosene ring system that is common to a number of antibiotics and antitumor agents. An overall 8 percent yield from hydroquinone was achieved (Equation 1). The key step of this exceptionally efficient synthesis is the palladium-catalyzed olefin-amination - olefin insertion process. Dr. Hegedus also developed a related, palladium(0) catalyzed approach to indoloquinones (Equation 2), and a very efficient approach to ergot alkaloids

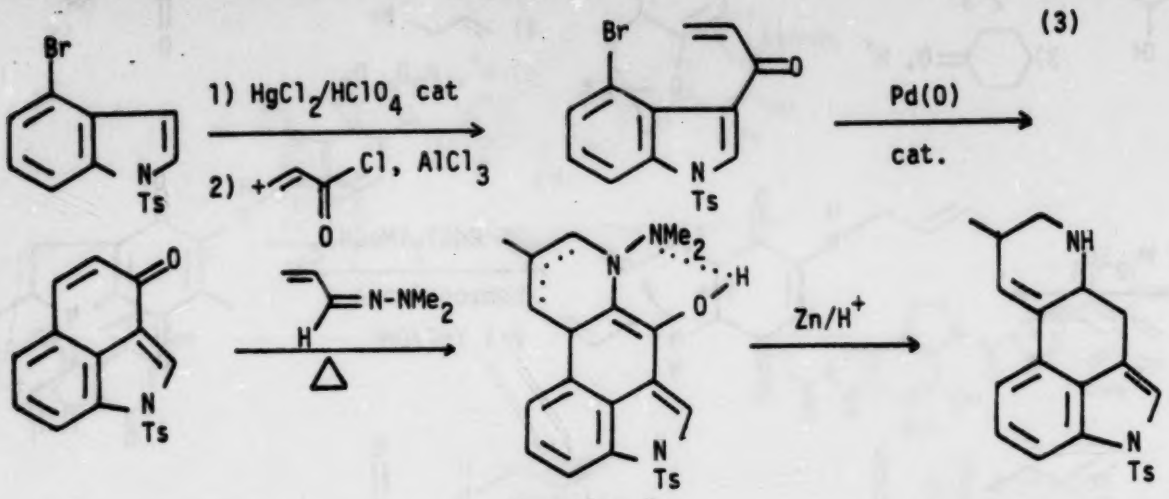


Equation 1



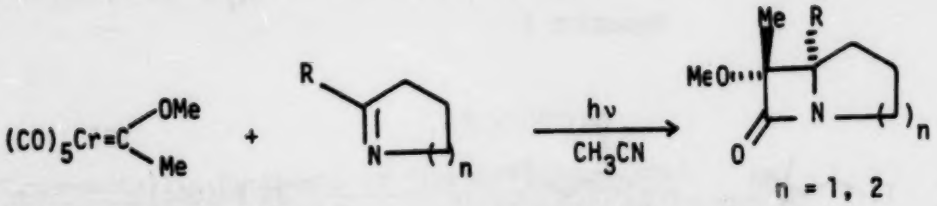
Equation 2

(Equation 3). The latter approach is very general and should permit the synthesis of a wide variety of differently-substituted biologically active ergot alkaloids.

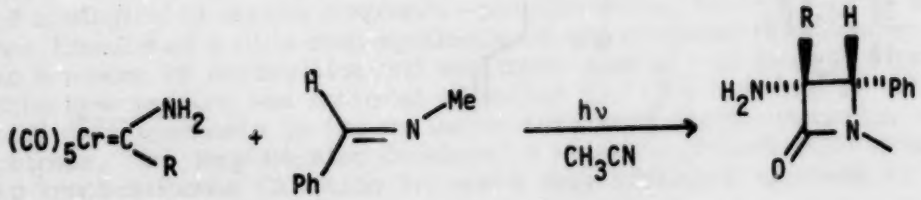


Equation 3

Finally, he recently synthesized carbapenam and carbacepham derivatives, as well as amino containing β-lactams, using the newly developed β-lactam synthesis from imines and chromium carbene complexes (Equations 4 and 5). This brings one step closer a new method for synthesizing natural and synthetic β-lactam antibiotics.



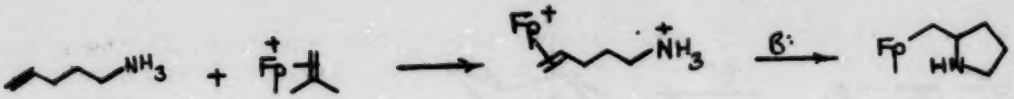
Equation 4



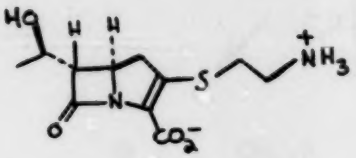
Equation 5

"Natural Product Synthesis Using Organoiron Reagents"
ROI GM 16395-15 (Rosenblum, M.), Brandeis University

Dr. Myron Rosenblum has employed organoiron reagents to develop a new, efficient synthetic route for β-lactams, important structural units of widely used antibiotics. This approach is illustrated below:

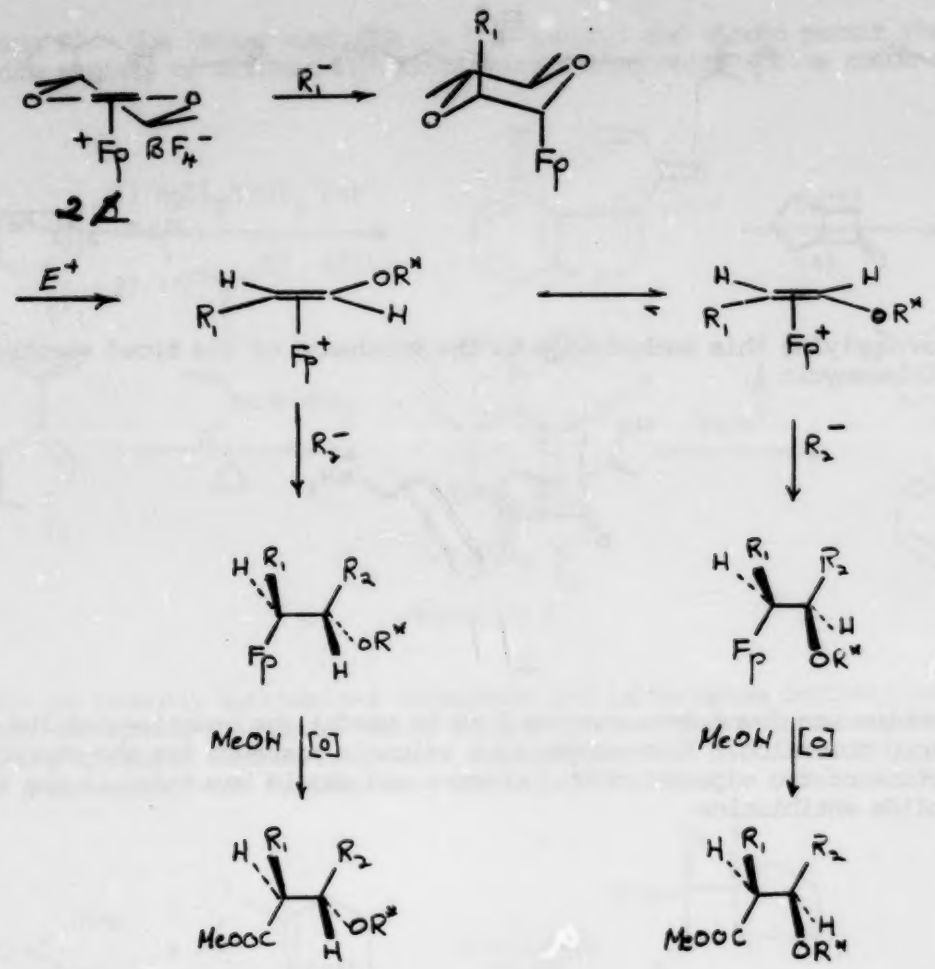


He is now applying this methodology to the synthesis of the broad spectrum antibiotic thienamycin 1.



1

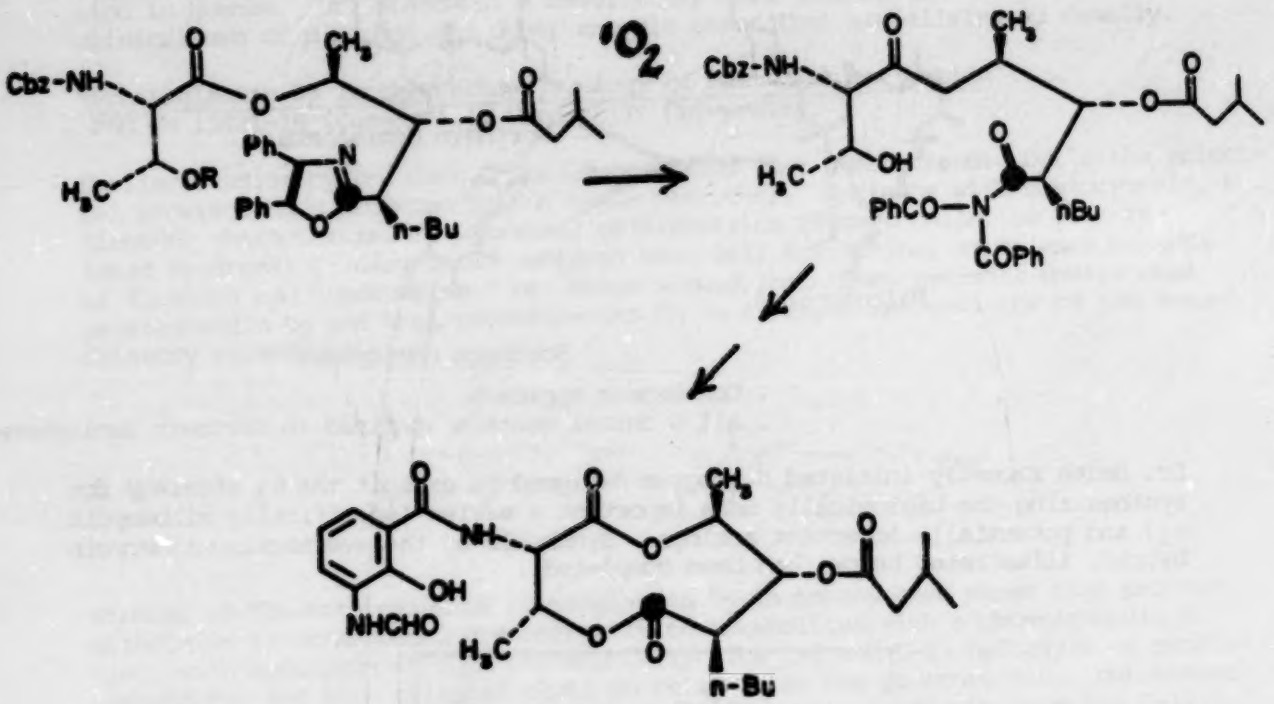
Dr. Rosenblum has found iron complex 2 to be useful for enantioselective carbon-carbon bond formation. This serves as a valuable platform for the stereocontrolled construction of two adjacent chiral centers and should be useful in the synthesis of macrolide antibiotics.



"Reactions of Molecular Oxygen with Organic Substrates"
R01 GM 13854-18 (Wasserman, H.), Yale University

Dr. Harry Wasserman has used singlet oxygen oxygenation as a key reaction for the macrocyclization required to synthesize (+)-Antimycin A_3. This naturally-occurring dilactone shows both antibiotic and antifungal activity.

The synthetic sequence makes use of the oxazole-singlet oxygen rearrangement as a mild, highly efficient pathway to an activated carboxylate derivative. As shown below, the oxazole provides the scaffolding for constructing the entire macrolide framework by stepwise substitution of chiral functionality at the site of a 2-methylene residue. Through a series of functional group changes, the latent 2 carboxyl group remains well protected, and the regeneration of this group as a triamide provides the activation for the final ring closure.

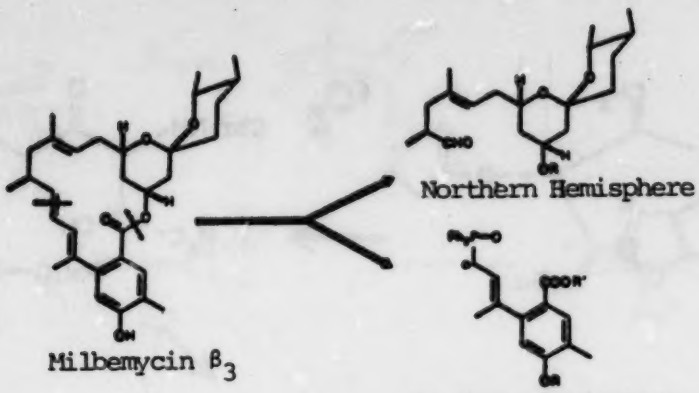


(+)-Antimycin A_3

"The Milbemycins--Potent Anthelmintic Agents"
R01 GM 29028-04 (Smith, A.), University of Pennsylvania

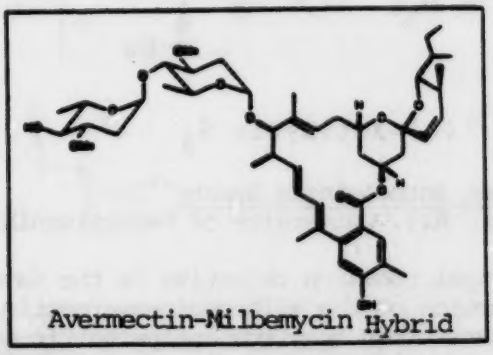
Dr. Amos Smith's principal research objective is the development of viable synthetic strategies for construction of the milbemycin-avermectin family of macrolide antibiotics, which are potent broad spectrum antiparasitic agents. He has achieved the first total synthesis of (±)-milbemycin β_3 and its epimer, epimilbemycin β_3, the simplest members of the milbemycin-avermectin class. His approach is relatively short (16 linear operations), convergent, and highly stereocontrolled. He has prepared 50 milligrams of milbemycin β_3 and its epimer and has submitted them to Merck, Sharp and Dohme (Rahway) for biological testing.

RETROSYNTHETIC ANAL YSIS OF MILBEMYCIN β_3



. Convergent approach
. All 6 chiral centers confined to Northern Hemisphere

Dr. Smith recently initiated a program designed to exploit the β_3 strategy for synthesizing the biologically more important α series (specifically milbemycin α_1) and potentially important analogs. Synthesis of the avermectin-milbemycin hybrid, illustrated below, has been completed.



Clinical Pharmacology

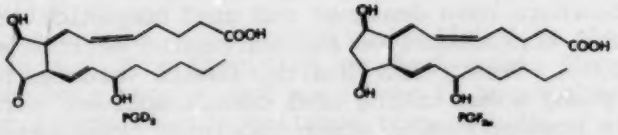
"Stereoselective Pharmacokinetics of Drugs in the Kidney"
R01 GM 31254-03 (Giacomini, K.), University of California, San Francisco

Stereoselective hepatic metabolism has been well documented both in vitro and in vivo, but very little is known about whether the kidney eliminates drugs stereoselectively. Dr. Kathleen Giacomini has used pindolol, a beta adrenoceptor-blocking agent that is marketed as a racemic mixture, as a model compound to investigate stereoselective renal clearance of basic drugs in humans. For this research, she developed a high performance liquid chromatographic procedure for separating and quantitating the enantiomers. In six normal volunteers studied, renal clearance of l-pindolol was greater than that of d-pindolol; when filtration was excluded, the net clearance by secretion of the l-enantiomer was about 40 percent greater than that of the d-enantiomer. Stereoselective binding of pindolol to plasma

proteins did not occur. The studies provide the first evidence that stereoselective renal excretion of organic bases may be an important route for drug elimination in humans. Dr. Giacomini's results may have general implications for the clinical use of pindolol and other racemic bases that are eliminated renally.

"Prostaglandin D_2 in the Pathophysiology of Mast Cell Activation"
P50 GM 15431-18 (Oates, J.), Vanderbilt University

Earlier studies by Dr. John Oates demonstrated that prostaglandin D_2 is the principal prostaglandin produced by the human mast cell. Patients with mastocytosis, a disorder characterized by increased proliferation of mast cells, or with related syndromes of disordered systemic mast cell activation, experience attacks of flushing and hypotension. Dr. Oates showed that these patients overproduce prostaglandin D_2 and that prostaglandin D_2 is an important mediator of the vasodilatory episodes.



Studies of the metabolism of prostaglandin D_2 in humans have shown that prostaglandin D_2 is transformed predominantly to metabolites with a prostaglandin F ring, with hydroxyls at both C-9 and C-11. The C-9 and C-11 hydroxyls of prostaglandin $F_{2\alpha}$ are both oriented alpha in relation to the prostane ring, but almost all of the prostaglandin F-ring metabolites of prostaglandin D_2 have the C-11 hydroxyl oriented in the beta position. Dr. Oates recently investigated the conversion of prostaglandin D_2 to prostaglandin F_2 by human liver. Prostaglandin D_2 was found to be converted essentially exclusively to prostaglandin F_2 by a cystolic NADPH-requiring enzyme in human liver. Stereochemical analysis indicated that this prostaglandin F_2 compound was $9\alpha,11\beta$-prostaglandin F_2 and not prostaglandin $F_{2\alpha}$. This finding established the unique structure of the initial product of prostaglandin D_2 metabolism.

Dr. Oates has demonstrated that $9\alpha,11\beta$-prostaglandin F_2 is a biologically active metabolite of prostaglandin D_2 that increases blood pressure in rats, contracts human coronary arteries and bronchial smooth muscle in vitro, and inhibits platelet aggregation. In view of these effects of $9\alpha,11\beta$-prostaglandin F_2, it was important to determine whether this compound is actually produced in vivo in humans. For this, Dr. Oates developed a mass spectrometric assay for quantifying $9\alpha,11\beta$-prostaglandin F_2. The compound is indeed present in plasma and urine in normal individuals. Furthermore, its level increases as much as 2,800-fold above normal in patients with mastocytosis during episodes of severe vasodilation.

The biologic properties of prostaglandin D_2 have been explored in a number of laboratories, yielding the findings that prostaglandin D_2 is a bronchoconstrictor and a potentiator of leukotriene B_4 chemotaxis. Recently, Dr. Oates found that prostaglandin D_2 in bronchoalveolar lavage fluid increases strikingly after antigenic challenge to the lung of asymptomatic patients with allergic asthma. Further studies that elucidate the full spectrum of biological activity for $9\alpha,11\beta$-prostaglandin F_2 and the sites of its formation should provide insights

into the pathophysiology of disorders associated with mast cell activation with attendant release of prostaglandin D_2.

"Immunopharmacokinetics-Immunopharmacodynamics"
P50 GM 26691-06 (Benet, L.), University of California, San Francisco
"Interindividual Variability in Oxidative Metabolism in Man"
P01 GM 31304-03 (Wilkinson, G.), Vanderbilt University
"Human Serum Organophosphatase"
P01 GM 27028-05 (Weber, W.), University of Michigan at Ann Arbor

Differences in the quantitative handling of a drug by oxidative metabolism often are a major contributor to the sometimes wide interpatient variability in drug responsiveness. These differences may be caused by one's physiological state, by other drugs one receives, or by one's heredity. Patients who metabolize a drug readily may receive inefficacious therapy from the administration of usual drug dosages, whereas poor metabolizers may have an increased risk of adverse effects, or when active metabolites are involved, a reduction in efficacy. Dr. Leslie Benet and his coworkers have designed and used companion studies to determine the effects of oral contraceptives and conjugated estrogens on the pharmacokinetics of prednisolone. Twenty-four healthy female volunteers entered the studies, including: six young women taking oral contraceptives, and six age-matched controls; and six postmenopausal women receiving conjugated estrogens, and six age-matched postmenopausal controls. Significant decreases in the clearance and volume of distribution and a significant increase in the half-life were observed for both total and unbound prednisolone in the women taking oral contraceptives, compared to the young controls. A significant decrease in the unbound clearance and increases in the total and unbound half-life were observed for prednisolone in the women receiving conjugated estrogens, compared to the postmenopausal controls. These companion studies showed that the unbound clearance of prednisolone was significantly lower in the young women taking oral contraceptives than in the postmenopausal women receiving conjugated estrogens, suggesting that part of the effect of oral contraceptives on prednisolone pharmacokinetics may be due to a general effect of estrogen. Thus, administration of prednisolone to women receiving estrogen-containing contraceptives or conjugated estrogens leads to higher than normal concentrations of unbound prednisolone for longer periods of time, which could increase the pharmacologic and toxic effects of prednisolone in these women.

Dr. Benet and coworkers also examined the immunosuppressive activity of prednisolone and its interconversion product prednisone, using the mixed lymphocyte reaction and mitogen stimulated cultures. They found that prednisone was inactive but could be converted to its biologically active 11-hydroxyl metabolite, prednisolone. These studies led Dr. Benet and his coworkers to examine the inhibitory effect of prednisolone on various stages of cell activation during the course of the mixed lymphocyte reaction. They found that lymphocytes are relatively insensitive to prednisolone before activation but that they become extremely sensitive to the drug's inhibitory effect immediately following cell activation. This sensitivity decreases with time and disappears after about 30 hours. These results are consistent with the hypothesis that prednisolone exerts its inhibitory effect by specifically interfering with the early phase of cell activation, prior to any significant increase in DNA synthesis by the cells.

Dr. Grant Wilkinson and colleagues at Vanderbilt University are studying the genetic basis for variability in drug metabolism, focusing particularly on polymorphisms involving the 4-hydroxylation of debrisoquine and mephenytoin. In collaboration with Japanese investigators at the University of Tsukuba, they have compared the frequency of impairment of these two independently inherited pathways. In Caucasians living in middle Tennessee, 8 percent were found to be "poor metabolizers" of debrisoquine, an incidence similar to that found in other populations of the same ethnic origin. In contrast, no individual was found in Japan who had this phenotype in over 100 consecutive, unrelated subjects. On the other hand, the frequency of impaired oxidation of the S-enantiomer of mephenytoin was sixfold higher in Japanese versus Caucasians, that is 18 percent and 3 percent, respectively. These pronounced interethnic differences in oxidative metabolism confirm a genetic independence of the two polymorphisms and support anecdotal and clinical experience that drug responsiveness often differs substantially between ethnic groups such as Orientals and Caucasians. Given the international nature of drug development and use and the multiracial populations of many countries, the findings indicate the need for increased recognition of such differences in drug evaluation.

Debrisoquine oxidative status is now known to be linked to the metabolism of over 30 different drugs ranging from tricyclic antidepressants through beta-adrenergic blockers to antiarrhythmic agents, but much less is known about the specificity of the S-mephenytoin polymorphism. In collaboration with Dr. F. P. Guengerich, Dr. Wilkinson has identified a number of other anticonvulsant drugs whose metabolism is thought to be controlled by the same genetic factor(s) as that of mephenytoin. This grouping was based on their ability to competitively inhibit the 4-hydroxylation pathway in human liver microsomes in vitro. A common feature for potent inhibition is the presence of an α-aryl-N-alkyl lactam substructure, e.g., ethotoin, methsuximide and mephobarbital. Clinical studies with mephobarbital confirmed the in vitro prediction that its 4-hydroxylation co-segregates with that of mephenytoin. Drs. Guengerich and Wilkinson also isolated, purified and characterized a specific cytochrome P-450 isozyme from human liver microsomes that is primarily responsible for the 4-hydroxylation of mephenytoin. The isozyme differs catalytically, electrophoretically and immunochemically from other human oxidative isozymes. Studies on isozymes such as this one will be useful for delineating the molecular and genetic regulation in man of cytochrome P-450 function.

The importance of a major gene effect in drug metabolism also was demonstrated during the clinical evaluation of a new antiarrhythmic agent, propafenone. Early studies indicated that the drug is efficacious but exhibits pronounced interpatient variability in disposition and effective plasma concentrations. Dr. Wilkinson and colleagues have now shown this to be due to the production of a pharmacologically active metabolite, 5-hydroxypropafenone, which is determined by the debrisoquine phenotype of the patients. Thus, poor metabolizers have unexpectedly higher plasma levels of unchanged drug and reduced concentrations of active metabolite. This could lead to an increased risk of adverse effects unless the interphenotypic difference in metabolism is taken into account.

Dr. Bert LaDu's research concerns the genetics of a relatively non-specific paraoxonase/arylesterase from human serum that catalyzes the hydrolysis of a number of organophosphate compounds (such as paraoxon, DFP, tabun, sarin, aramine), carbamates (carbaryl, 3-isopropylphenyl-N-methylcarbamate) and aromatic carboxylic

acid esters (phenylacetate, 4-nitrophenylacetate, 2-naphthylacetate). It requires calcium for activity and is inhibited by SH-binding agents, such as p-chloromercuribenzoate. Paraoxonase activity appears to be polymorphic, with some individuals in a sample population showing "high" paraoxonase activity and others showing "low" activity. Dr. LaDu has devised a new method for subdividing individuals into three groups: A (low activity), AB (intermediate), B (high). Standard genetic analytical tests performed with 37 family pedigrees yielded convincing evidence that two autosomal genes at one locus determine serum paraoxonase. The estimated gene frequencies are 0.685 for A and 0.315 for B. This would mean that about 9.9 percent of the people in the United States are B type, 43.2 percent are AB, and 46.9 percent are A type. Similar figures have been found with sample populations of English, Canadian, German, Dutch, and French people, but much higher B frequencies have been reported for some African, Far Eastern, and Asian groups. The average B type person has about seven times as much paraoxonase activity as the average A type person.

Dr. LaDu has purified human organophosphatase 200- to 300-fold from both A-type and B-type serum. He found the qualitative differences between the purified enzymes that were observed earlier in serum samples were unchanged by purification. The selective stimulation by NaCl, differences in pH optima, different ratios of activity with paraoxon vs. phenylacetate, and the different degree of inhibition by phenothiazines all are consistent with the hypothesis that "high" and "low" paraoxonase activities are due to isozymic, qualitatively different but closely related enzymes and not to a quantitative difference in the level of the same enzyme.

Several experiments were performed to determine whether having either A or B type of organophosphatase activity should influence a person's susceptibility or resistance to paraoxon exposure. Computer modeling, measurements of paraoxon pharmacokinetics in an in vivo rabbit model, and in vitro studies with rabbit serum and human blood and serum samples of the A and B type have all supported the hypothesis that individuals with B type enzyme should be more resistant to the effects of organophosphates such as paraoxon than A type people.

Human serum organophosphatase/paraoxonase activity seems to be associated with the HDL (high density lipoprotein) complex; none of it is free in the blood. This could suggest a role of the enzyme in endogenous lipoprotein metabolism.

Pharmacokinetics

"Pharmacokinetics and Pharmacodynamics—Developmental Considerations"
P50 GM 26691-06 (Benet, L.), University of California, San Francisco

Considerable controversy has existed regarding the disposition of the glucuronide and sulfate conjugates of acetaminophen by the fetus. Drs. L. H. Wang, Abraham Rudolph, and Leslie Benet have found that although acetaminophen itself is readily transferred across the placenta from mother to fetus and fetus to mother, there was no detectable transfer of glucuronide or sulfate conjugates of acetaminophen. Fetal elimination of the acetaminophen conjugates could be accounted for largely by renal excretion. However, because renal function is relatively immature during fetal life, plasma concentrations of the conjugates remain at high levels for prolonged periods, compared to the adult. Although the acetaminophen conjugates

have no known pharmacological activity or toxicity, the observation that they do not appear to be transferred across the placenta may have important clinical implications. Thus, drugs administered to the mother that enter the fetal circulation could exert prolonged effects on the fetus through active conjugates long after they have been eliminated by the mother.

"Renal Transport of Cimetidine and Ranitidine"
P50 GM 26691-06 (Benet, L.), University of California, San Francisco

Cimetidine, an H_2-receptor antagonist, is excreted by the kidney. Previous studies in several species suggested that this substance has complex secretion characteristics that cannot be attributed exclusively to transport by the organic cation transport system. Dr. Kathleen Giacomini has prepared luminal membrane vesicles from rabbit renal cortex and has used them to study the renal transport of cimetidine. Her data indicate that the drug is transported across the luminal membrane via two transporters. One transporter appears to be the organic cation transporter and is inhibited by classical inhibitors of organic cation transport. The second transporter of cimetidine, which had not been described previously, can be inhibited by probenecid and appears to be distinct from the organic anion transporter.

"Drug Interactions Affecting α1-Acid Glycoprotein Levels"
R01 GM 30529-03 (Abramson, F.), George Washington University

Dr. Fred Abramson has developed several models for enzyme induction that can be used to identify the aspect of the kinetics that is altered by the inducer, such as direct stimulation of synthesis, stabilization of product, or stabilization of precursor. These models can be used to evaluate kinetic lag phases that normally are ignored in other kinetic schemes since the new model includes the kinetics of the inducing drug in describing the overall time course of the induced substance. This aspect has never before been incorporated into an interpretive model.

At least two rate constants are needed to describe the time course of concentration changes of an induced substance while the inducer is being given or after it has been withdrawn. These are the turnover rate constant for the induced substance and the elimination rate constant for the inducing substance. As long as the elimination rate constant is rapid compared to the turnover rate constant, the time course of the induction or the deinduction period is governed solely by the turnover rate constant. The slope of a semilog plot of the induced substance against time yields the turnover rate constant. If the elimination rate constant is not at least four times larger than the turnover rate constant, the slope will be affected significantly by the elimination rate constant.

A third rate constant may have to be considered if lag phases in induction and deinduction are observed that are not caused directly by inducer accumulation or decline. Periods where growth or decay are inappropriately slow probably are caused by the accumulation or decline of a precursor of the induced substance, such as a specific mRNA associated with the induced product. Unless precursor turnover is rapid compared to the turnover rate constant, the kinetics of the synthesis precursor may play a dominant role in determining the rates of growth and decay of the induced substance. This leads to a nonlinear kinetic pattern that could not be interpreted with previously developed models.

Dr. Abramson has used these equations to fit data for the induction of α1-acid glycoprotein by rifampin in dogs. The applicability of the model is seen by its ability to fit the data via computer in a continuous fashion, from onset of induction through the relaxation period. The nonlinear nature of α1-acid glycoprotein induction is manifest by a lag period when the inducer is first given and another lag period preceding decay once the inducer is withdrawn. The coefficient of determination (r^2) was 0.95.

Dr. Abramson also has evaluated the kinetics by which the turnover rate constant for the induced substance or its synthesis precursor is reduced. This is conventionally called "stabilization" of a product. The kinetics of this situation are unique, thus permitting this mechanism to be differentiated from all other mechanisms.

Overall, these kinetic descriptions provide a much better tool than previous theories for interpreting induction experiments. They evaluate each of the several steps where rate constants other than the one whose measurement is being sought may affect induction behavior.

"Pharmacokinetic/Pharmacodynamic Modelling in Data Analysis"
P50 GM 26691-06 (Benet, L.), University of California, San Francisco

Combined pharmacokinetic and pharmacodynamic data from non-steady-state experiments require the application of three models connected in series for their full description. A pharmacokinetic model relates dose to observed drug concentrations in plasma; a link model relates drug plasma concentrations to drug concentration at the effect site; and a pharmacodynamic model relates drug concentration at the effect site to the observed effect. Parametric models can be used for all three, but they require considerable assumptions. Dr. Lewis Sheiner and coworkers recently suggested the use of non-parametric pharmacodynamic models and have extended this approach to both pharmacodynamic and pharmacokinetic submodels. To test this more assumption-free approach, he has submitted data on drug plasma concentrations from several commonly encountered pharmacokinetic models and effect data from several pharmacodynamic models, added noise, and analyzed the resulting data by the fully parametric approach and by the largely non-parametric one.

He also generated data on drug plasma concentrations from one pharmacokinetic model and then fit them in the parametric approach to a distinct, but plausible, other model to assess the effect of model misspecification on the parametric approach. An average measure of the error in predicting effect was computed for each method, for over 100 simulations. As expected, in the absence of model misspecification, the non-parametric method loses some precision: its error is 0-6.6 percent greater than that of the fully parametric approach. However, in the presence of model misspecification, the non-parametric method is more precise: its error is 0-7.7 percent less than that of the parametric method. Since one can never be certain that model misspecification is absent, the non-parametric approach offers a distinct advantage for routine application in drug modelling analysis.

"New Ways to Investigate and Forecast Pharmacokinetics"
R01 GM 26676-06 (Sheiner, L.), University of California, San Francisco

Calibration is a familiar and sometimes costly problem. It generally is performed by assaying samples containing known concentrations of analyte, and these points are used to construct a "standard curve," from which unknown analyte concentrations in subsequent samples may be estimated. Calibration curves involving N parameters might appear to need a minimum of N calibrators; yet curves may be relatively stable, suggesting that fewer calibrations might suffice. Dr. Lewis Sheiner has applied an Empirical Bayesian method to the calibration problem and has compared it to the familiar Least Squares method. The Empirical Bayesian method uses the calibrators plus estimates of the mean and co-variance of the standard curve parameters and an estimate of the residual error in the curve fits (obtained from historical standard curve data) to estimate current standard curve parameters. The historical mean and co-variance is estimated using NONMEM, a system for estimating population parameters. The Least Squares method uses only the calibrators. When their number, p, is less than N, it fixes N-p parameters to their historical mean value. In comparing the two methods using both real and simulated data, the average error (in "unknown" analyte values) for a four-parameter (logistic) calibration curve, using one optimal calibrator, was 30 percent less than that for the Least Squares method; if two optimal calibrators were used, the average error was 10 percent less than that for the Least Squares method. Under realistic conditions, using a single calibrator and the Empirical Bayesian method, ultimate assay error was less than 5 percent. The Empirical Bayesian method would be most useful when (i) run-to-run variability in standard curve parameters is small, yet large enough to warrant regular calibration; (ii) the residual error is significant; and (iii) there is a desire to use few calibrators.

Drug Toxicity and Toxins

"Hepatic Microsomal Enzyme Metabolism of Anesthetics"
R01 GM 23029-07 (Kaminsky, L.), New York State Department of Health

Previous studies showed that 2,2,2-trifluoroethanol is the cytochrome P-450 catalyzed metabolite of the volatile anesthetic agent fluroxene and its ethyl and vinyl analogs, and that this metabolite is responsible for the lethality of fluroxene in rats.

Recent studies by Dr. Laurence Kaminsky reveal a novel and highly complex mechanism whereby the metabolite trifluoroethanol expresses its lethal effects. Trifluoroethanol produces marked edema in the small intestine and ileocecal junction, and the associated lesions permit bacterial infection. It also markedly decreases the bone marrow-nucleated cellularity. As a consequence, the peripheral leukocyte count decreases to 15 percent of control values and infecting bacteria are able to proliferate. Trifluoroethanol appears to have a direct effect on bone marrow cells, as was demonstrated by in vitro studies with bone marrow cell suspensions. In vivo studies also show that trifluoroethanol synergistically enhances the lethality of bacterial endotoxins approximately 1000-fold. The clinical symptoms associated with death from trifluoroethanol generated metabolically from fluroxene are very similar to those associated with a lethal dose of endotoxin. Metabolically produced trifluoroethanol thus causes lesions which permit bacterial infection, destroys the host defenses against the bacteria, and synergistically

enhances the lethal effects of the endotoxins. This complex mechanism of action for such a relatively simple molecule may provide insight into the toxic effects of many other halogenated xenobiotics.

"A Biochemical Characterization of Conus Toxins"
R01 GM 22737-07 (Olivera, B.), University of Utah

A change in cytoplasmic calcium levels is a general mechanism for signaling a change in cellular physiology, particularly in the nervous system. At the presynaptic termini of nerves, the action potential is coupled to the release of neurotransmitter by a rise in calcium levels at the presynaptic terminus. This occurs via a voltage-activated calcium channel located at nerve presynaptic termini. Dr. Baldomero Olivera has identified, isolated, purified, and characterized a group of peptide toxins, the ω-conotoxins, that inhibit voltage activated calcium channels. To date, he has isolated eight different but homologous peptides that belong to the ω toxin class. The best studied of these Ca^{+2}-channel inhibitors is a 27 amino acid basic, cystine-rich peptide from Conus geographus venom. Electrophysiological experiments show that this peptide irreversibly blocks transmission in all frog synapses tested.

The ω-conotoxins appear to fall into two separate classes based on sequence homology. Class A ω-conotoxins inhibit only the neuromuscular junction of fish. However, class B ω-conotoxins have a broader phylogenetic range: they inhibit the synapses of fish, amphibians, and birds. Neither class A nor class B ω-conotoxins inhibit mammalian neuromuscular junctions, but both classes cause central nervous system symptoms in mice. The response is a Parkinsonian-like syndrome of prolonged shaking. Thus, the mammalian neuromuscular junction calcium channel seems not to be recognized by either type A or type B ω-conotoxins, but an ancestral voltage-activated calcium channel may be present in the central nervous system that is recognized by both types of ω-conotoxins.

In addition to the ω toxins described above that block voltage activated calcium channels at the presynaptic terminus, conus venoms also contain peptides that block the acetylcholine receptor of the neuromuscular junction and other peptides that block muscle sodium channels. Dr. Olivera also has found accessory peptides that may assist the major toxins to reach their target tissues as part of the general strategy of paralysis by predatory cone snails. One of these peptides is a novel vasopressin that has been purified, characterized and chemically synthesized. This vasopressin has a number of unique features but is clearly homologous to the neurohypophyseal hormones. Peptides of this type elicit scratching when injected intracutaneously into mice. A number of additional scratcher peptides have been isolated and are being characterized structurally.

"Characterization and Inhibition of Snake Hemorrhagic Toxins"
R01 GM 31289-03 (Fox, J.), University of Virginia Medical School
"Hemorrhagic and Necrotic Toxins in Snake Venoms"
R01 GM 15591-17 (Tu, A.), Colorado State University

Dr. Jay Fox is investigating the structure and substrate specificity of hemorrhage-producing toxins in rattlesnake venoms. Recently, he has concentrated his efforts on analyzing the site preference for protein digestion by two of the less potent toxins isolated from the venom of the Western diamondback rattlesnake, Crotalus atrox. Using the oxidized B chain of insulin as a substrate, each of the two

toxins was able to cleave the peptide at five identical sites. The toxins preferred to cleave X-Leu peptide bonds but also could cleave the Gly-Phe bond. In order to determine the minimum length substrate necessary for the toxins, Dr. Fox synthesized peptide substrates of varying size and assayed them for cleavage by the toxins. Both toxins preferred longer substrates and would not cleave a tetrapeptide. Dr. Fox probed the substrate binding sites of the enzymes by synthesizing peptide substrates with various amino acid residues at positions around the peptide bond to be cleaved. The enzymes preferred peptide bonds in which the amino terminal portion was donated by small aliphatic amino acid residues and the carboxyl terminal donated by larger aliphatic residues. Dr. Fox found that hydroxamic amino acids inhibit the hydrolytic activity, with inhibition constants in the low 10^{-4}M range. These inhibitors take advantage of the ability of the amino acid side chain moiety to bind to the enzymes' substrate binding site, while the hydroxamic acid moiety binds to the active site zinc. Dr. Fox is using this information to design and synthesize snake toxin inhibitors that have greater specificity and that might be useful clinically to treat snake bite.

Venom from Crotalidae snakes (pit vipers) causes hemorrhage and strongly affects the blood coagulation system. These venoms contain both procoagulant and anticoagulant components, along with a variety of hemorrhagic toxins, that can be isolated by liquid chromatography. It had been believed that the hemorrhagic and anticoagulant activities were caused by completely unrelated components. However, recent work by Dr. Anthony Tu shows that several anticoagulants, with or without hemorrhagic activity, are capable of hydrolyzing fibrinogen, fibrin, or both. Some hydrolyze the A chain of fibrinogen first and then the B chain, while still other anticoagulants hydrolyze either the A or B chain, but not both. Thus, several snake venom proteases can have diverse physiological roles capable of inducing either anticoagulation or hemorrhage.

"Sea Anemone Toxin Action on Nerve Sodium Channels"
R01 GM 32848-01 (Kem, W.), University of Florida

Hydroxyproline residues recently have been found in several peptides and proteins unrelated to collagen, but their functional roles are as yet unknown. Dr. William Kem has devised a new method for identifying hydroxyproline in proteins. He prepared phenylthiohydantoin derivatives of trans-4-hydroxy-L-proline and trans-3-hydroxy-L-proline and determined their electron impact mass spectra. The 4-hydroxyproline derivative displayed a prominent molecular ion at m/e 248. The 3-hydroxy-L-proline derivative was much less stable; its most prominent ion occurring at m/e 230 indicates facile dehydration of this derivative. Dr. Kem used this approach to show that the neurotoxin from the sea anemone Condylactis gigantea possesses trans-4-hydroxy-L-proline at position 3.

Dr. Kem also has purified and determined the amino acid sequence of an arthropod-selective polypeptide from the sea anemone Stoichactis helianthus. Stoichactis neurotoxin is quite different from the five previously sequenced neurotoxins; less than 30 percent of its amino acid residues are the same as in the other anemone toxins. Most of the identical residues are implicated in maintenance of secondary and tertiary structure, including six half-cystinyl residues that form disulfide crosslinks and several B-turn forming residues. This finding suggests that sea anemone polypeptide sequences are much less conserved than initial investigations had suggested.

"Biochemistry of Cyanide Detoxication with Sulfane Sulfur"
R01 GM 30971-02 (Westley, J.), University of Chicago

Dr. John Westley is examining the mechanisms for sulfur-mediated cyanide detoxification. Radioisotopic labeling experiments with ^{35}S and light-scattering studies have established that each serum albumin molecule can bind up to four sulfur atoms in vitro. Cyanolysis studies have shown that the four sulfane sulfur atoms bound have different characteristic reactivities. The atom that reacts most rapidly with cyanide is at a site that can also be reloaded rapidly with sulfane sulfur. The result is an efficient catalytic mechanism for cyanide detoxification when an adequate supply of the right form of sulfur is available. Kinetic analysis of the albumin-catalyzed reaction of cyanide anion with elemental sulfur has established that the sulfur substrate involved in detoxifying cyanide is not the principal molecular species present in colloidal elemental sulfur, but is a quantitatively minor component that is generated continuously by a bimolecular process in colloidal sulfur suspensions. Understanding this system has potential for enhancing the natural detoxifying mechanisms for nucleophilic poisons.

Drug Metabolism

"Drug- and Xenobiotic-Metabolizing Enzyme Systems In Situ"
R01 GM 33253-02 (Baron, J.), University of Iowa

In collaboration with Dr. F. P. Guengerich at Vanderbilt University and Dr. W. B. Jakoby at the NIH, Dr. Jeffrey Baron has utilized a combination of histochemical and immunohistochemical methodologies to investigate at the light microscopic level the localization and distribution of a number of xenobiotic-metabolizing enzymes and aryl hydrocarbon hydroxylases. His recent studies have focused on the respiratory tract, as various portions of it are major targets for many carcinogens and other toxic chemicals. Immunohistochemical analyses demonstrated that cytochromes P-450 PB-B, BNF-B, and PCN-E (which are the major isozymes of rat hepatic microsomal cytochrome P-450 that are induced by phenobarbital, β-naphthoflavone, and pregnenolone-16α-carbonitrile, respectively), NADPH-cytochrome P-450 reductase, epoxide hydrolase, and glutathione S-transferases B, C, and E are each localized within bronchial and bronchiolar epithelial cells and alveolar wall cells in the lungs of untreated rats. The nonciliated bronchiolar epithelial cell, the Clara cell, contains the greatest amount of each of these enzymes. Benzpyrene hydroxylase activity also was detected within bronchial and bronchiolar epithelia and alveolar walls, the greatest activity being in the Clara cells. Thus, the Clara cell appears to be the major site for the in situ metabolism of xenobiotics in the lung, but other pulmonary cells also can participate.

In pioneering studies of nasal tissue, Dr. Baron found benzpyrene hydroxylase activity and cytochromes P-450 PB-B, BNF-B, PCN-E, and MC-B (the major isozyme of rat hepatic microsomal cytochrome P-450 induced by 3-methylcholanthrene), NADPH-cytochrome P-450 reductase, epoxide hydrolase, and glutathione S-transferases B, C, and E within the olfactory and respiratory epithelia, duct and acinar cells of Bowman's glands in the olfactory region, and duct and acinar cells of seromucous glands in the respiratory region.

"Hepatic Metabolism of Drugs, Toxins, and Environmental Pollutants"
R01 GM 33142-02 (Zakim, D.), Cornell University Medical Center

Fatty acids have two key characteristics in common with many xenobiotics: they are metabolized by enzymes that are integral components of membranes, and both fatty acids and many xenobiotics are relatively insoluble in water but are very soluble in lipids. Dr. David Zakim has found that failure to consider how fatty acids are solvated in cells has led to erroneous conclusions about the kinetic properties of microsomal long chain acyl CoA ligase, which uses fatty acid substrates from C_{12} to C_{18}. In earlier studies, this enzyme was assayed by adding fatty acids directly to an aqueous system. Detergents were used to disperse the fatty acids, or they were dissolved in an organic solvent. Since Dr. Zakim's finding that fatty acids bound to lipid bilayers become hydrated rapidly, he has assayed microsomal acyl CoA ligase by adding fatty acids as a component of the membranes of unilamellar lipid vesicles. The assay procedure yields ligase activity that is 10 to 40-fold higher than activities reported in the literature but reveals no direct effect of phospholipids on the ligase. Thus, the activity of the ligase has been underestimated because of a failure to consider the physical state of fatty acid in the assay system versus the physical state of fatty acid under physiologic conditions. With his assay system, Dr. Zakim found that ligase activities at V_{max} are higher for shorter chain fatty acids than for longer chain fatty acids, and higher for unsaturated than for saturated fatty acids. K_m values appear to depend on how the fatty acid interacts with the lipids of the microsomal membrane. An important insight into this specific system that appears to have general application to the metabolism of water-insoluble substrates by membrane-bound enzymes is that reaction [2], not [1], describes the interactions of ligase with fatty acids.

The rate of spontaneous transfer of fatty acids from unilamellar lipid vesicles

$$[1]\quad FA\bullet(H_2O)n + E \rightleftharpoons E\ FA$$

$$[2]\quad FA\bullet(\text{membrane PL}) + E \rightleftharpoons E\ FA$$

to microsomes, a hydrated intermediate, is far more rapid than the inherent catalytic activity of the ligase (enzyme saturated with ATP, CoA and fatty acids). Putative fatty acid binding proteins that activate the ligase when the enzyme is assayed with fatty acids added directly to water were found instead to inhibit ligase activity when fatty acids are components of unilamellar lipid vesicles. This raises serious questions about the physiologic functions of these proteins. There is a direct correlation between rate constants for hydration of different fatty acids bound to unilamellar vesicles and K_m. Since rates of solvation of fatty acids appear to be limited by the escape of fatty acids from solvation by membrane lipids and not by entry into water, solvation forces for fatty acids in membranes (and for other substances dissolved in membranes) may be important determinants of the equilibrium position of reaction [2].

"The Role of Epoxides in Metabolism and Drug Toxicity"
R01 GM 21784-10 (Hanzlik, R.), University of Kansas, Lawrence

The hepatotoxicity caused by bromobenzene administration has been associated with the covalent binding of chemically reactive metabolic intermediates to vital cellular macromolecules. Epoxide metabolites, particularly bromobenzene-3,4-oxide,

have been suspected of being responsible for the covalent binding and toxicity observed, although recently a role for secondary metabolites such as quinones has been advocated. Dr. Robert Hanzlik has carried out two types of studies which provide considerable additional support for the latter. In one study the effects of 26 different chemical or enzymic probes on the in vitro metabolism and covalent binding of bromobenzene were determined. As expected, P-450 inhibitors caused strictly proportionate decreases in both metabolism and covalent binding. With various antioxidants, reducing agents, superoxide dismutase and bromophenols, inhibition of covalent binding was only slightly greater than their inhibitory effect on metabolism. However, sulfur nucleophiles (e.g., thiourea, bisulfite ion, mercaptoethanol, N-acetylcysteine or glutathione) very strongly and selectively suppressed covalent binding, with little or no effect on bromobenzene metabolism. The remarkable efficacy of thiourea and bisulfite are consistent with a major role for quinones in the covalent binding of bromobenzene under in vitro conditions.

Further support for this, as well as insights into the chemistry of covalent binding of bromobenzene in vivo, was provided by experiments with tritium/carbon-14 dual-labeled bromobenzene. Under in vitro conditions, only 25-30 percent as much tritium as carbon-14 becomes covalently bound to microsomes and a considerable amount of tritiated water is produced. N-acetylcysteine inhibits covalent binding by 65 percent, with no effect on the total amount metabolized or on the tritium/carbon ratio of the bound material. This extensive loss of tritium is not compatible with a major role for epoxide metabolites in the covalent binding of bromobenzene in vitro, but it is consistent with a role for secondary metabolites such as quinones. When the tritium/carbon ratios of covalent binding material in tissues was measured 4 hours after administration of tritium/carbon dual-labeled bromobenzene to rats, the results for whole liver, S-12 and liver microsomes were identical to those observed in vitro, although significantly higher tritium/carbon ratios (ca. 0.55) were observed in lung and kidney. Thus, in vitro studies such as those described here seem to provide useful information on liver metabolism of bromobenzene in vivo and strongly support a major role for quinones in the covalent binding of bromobenzene metabolites.

"Endocrine Effects of Spironolactone: Role of Metabolism"
R01 GM 30261-02 (Colby, H.), West Virginia University

Spironolactone is a renal mineralocorticoid antagonist that is widely used for treating essential hypertension, congestive heart failure, and other edematous states. The drug causes several side effects, including interference with adrenocortical, testicular, and hepatic function. Indirect evidence indicates that both the therapeutic actions and side effects of spironolactone are caused by metabolites of the drug. In endocrine tissues like the adrenal cortex and testes, spironolactone causes the destruction of cytochrome(s) P-450, thereby decreasing the activities of several steroidogenic enzymes that are required for hormone biosynthesis.

Previous investigations indicated that spironolactone was converted to 7α-thio-spironolactone by microsomal preparations from kidney, adrenal cortex, testes, and liver. While that conversion did not require NADPH, the destruction of cytochromes P-450 in adrenal cortex and testes was NADPH-dependent and was accompanied by the further metabolism of spironolactone beyond 7α-thio-spironolactone. NADPH had no effect on spironolactone metabolism in tissues not adversely affected by the drug. Thus, the NADPH-dependent metabolites may be responsible for the

destruction of cytochromes P-450. However, the metabolites produced in the presence of NADPH were not detectable by UV absorbance or by fluorescence and, therefore, were not readily detectable by high performance liquid chromatography. To further pursue the metabolism of spironolactone, Dr. Howard Colby prepared radiolabelled spironolactone by synthesizing the appropriate precursor and then reducing it with tritium gas to yield 1, 2-^{3}H-spironolactone of high specific activity. The compound is more than 99 percent pure as determined by high pressure liquid chromatography. Liver microsomes readily convert ^{3}H-spironolactone to ^{3}H-7α-thiospironolactone.

When ^{3}H-spironolactone and ^{3}H-7α-thio-spironolactone are incubated with adrenocortical or testicular microsomes in the presence of NADPH, they are converted to water-soluble metabolites and metabolites that covalently bind to microsomal protein. Those metabolites are not produced by hepatic or renal microsomes. The effects of glutathione on spironolactone metabolism in adrenal cortex and testes suggest that the metabolites may include the sulfenic acid derivative of 7α-thio-spironolactone, a highly reactive compound. This information may lead to a better understanding of how drugs are metabolized and may contribute to the development of drugs with greater therapeutic value and fewer adverse effects.

"Interaction of Imidazole Derivatives and Epoxide Hydrolase"
R01 GM 32547-01 (James, M.), University of Florida

Epoxides and arene oxides are potentially cytotoxic metabolites that are formed in vivo by the cytochrome P-450 dependent monooxygenation of molecules containing double bonds or aromatic rings. The microsomal enzyme epoxide hydrolase plays a key role in metabolizing reactive epoxides and arene oxides to dihydrodiols which are almost always of low toxicity. Previous research by Dr. Margaret James and others has shown that some molecules which contain the imidazole ring enhance epoxide hydrolase activity in vitro with some epoxide substrates. Thus, imidazole-containing drugs could "direct" the metabolism of certain other xenobiotics to non-toxic products. Dr. James is studying the structural features in both the imidazole derivative and the epoxide substrate which are needed in order for the enhancement of activity to occur. She finds that imidazole derivatives with large, lipophilic substituents at the 1-position of the imidazole ring are most potent for enhancing epoxide hydrolase activity with styrene oxide as substrate. Some widely used antifungal drugs fall into this structural category. She also has shown that one or more microsomal membrane components are needed for the enhancement of epoxide hydrolase to occur.

"Alteration of Metabolism by Structural Modification"
R01 GM 25418-07 (Nelson, S.), University of Washington

Dr. Sidney Nelson showed previously that N-acetyl-p-benzoquinone imine is a toxic metabolite of the widely used analgesic-antipyretic, acetaminophen, and that cytochrome P-450 can catalyze the oxidation of acetaminophen to N-acetyl-p-benzoquinone imine. Although cytochrome P-450-mediated oxidation is largely responsible for formation of the toxic metabolite in the liver, prostaglandin synthase may be the more important oxidative catalyst in other tissues such as the kidney. Ram seminal vesicle microsomes, a rich source of prostaglandin synthase, catalyze the formation of both N-acetyl-p-benzoquinone imine and radical recombination products, indicating the presence of potentially toxic 2- and 1-electron oxidation

products. Inhibition studies showed that the hydroperoxidase component of prostaglandin synthase is responsible for the activation of acetaminophen.

Relatively low concantrations of acetaminophen (0.2mM) stimulate the conversion of prostaglandin G_2 to prostaglandin H_2, whereas higher concentrations (>10mM) inhibit the formation of prostaglandins from arachidonic acid. Several analogs of acetaminophen have this effect; the order of potency with regard to both stimulation of prostaglandin H_2 synthesis and inhibition of prostaglandin synthesis is correlated with the electrochemical half-wave potentials of the compounds. Therefore, it may be difficult to prepare an analog of acetaminophen that is a good inhibitor of prostaglandin synthase, and thus retains therapeutic activity, but which is less capable of being oxidized to reactive metabolites.

Sometimes it is useful to generate reactive metabolites. For example, aromatase is a cytochrome P-450 enzyme complex that catalyzes the oxidation of the male hormonal androgens to the female hormonal estrogens. Thus, inhibition of aromatase can limit estrogen production, which is of therapeutic benefit in treating cancers that are dependent on estrogens for growth. Dr. Nelson has made minor structural modifications to the hormonal steroid, androstenedione, in attempts to develop a suicide inhibitor for the enzyme. His research group has prepared a 10-mercapto analog, which is isosteric with androstenedione, and a 19-mercapto analog, which is isoelectronic with the first oxidation product formed by aromatase oxidation of androstenedione. Both analogs are potent suicide inhibitors of human placental aromatase, probably via their oxidation to reactive sulfenic acids that bind to the active site of the enzyme.

"Drug Interactions"
P01 GM 32165-02 (Trager, W.), University of Washington

Drs. Rene Levy and Thomas Baillie have studied the metabolism of valproic acid in rat liver microsomal preparations. Incubation of valproic acid with these microsomal preparations leads to the formation of 3-, 4- and 5-hydroxy valproic acid. The latter two metabolites were characterized previously from in vivo studies and were regarded as products of fatty acid ω-1 and ω-hydroxylation, respectively. However, 3-hydroxyvalproic acid had been thought to be produced by the β-oxidation pathway in mitochondria. These investigators now find that conversion of valproic acid to all three metabolites in microsomes requires NADPH (NADH was less effective), utilizes molecular oxygen, is suppressed by inhibitors of cytochrome P-450 and is stimulated (notably at C-3 and C-4) by phenobarbital pretreatment of the rats. He has concluded that rat liver microsomal cytochrome P-450 catalyzes the ω-2-hydroxylation of valproic acid, a reaction that had not been detected previously with fatty acids in mammalian systems.

Cytochrome P-450

"Structure and Regulation of Cytochrome P-450 Genes"
R01 GM 30701-04 (Adesnik, M.), New York University
"Molecular Mechanisms of Expression of Cytochrome P-450"
R01 GM 32281-01 (Omiecinski, C.), University of Washington

The cytochrome P-450 system consists of at least a dozen distinct forms or isozymes, many of which individually exhibit broad, albeit distinguishable, substrate

specificities. Some forms of the enzyme are devoted primarily to the metabolism of foreign compounds, while other forms of the enzyme are devoted primarily to the processing of endogenous substrates, such as fatty acids, cholesterol, prostaglandins, leukotrienes, steroid hormones, and bile acids.

Dr. Milton Adesnick is using recombinant DNA techniques to study the structures and interrelationships of various cytochrome P-450 genes, as well as the mechanisms of their regulation. He has cloned cDNAs for the two major phenobarbital-induced P-450 isozymes of rat liver (P-450b and P-450e), the corresponding gene for one of them (P-450e), and five other related cross hybridizing genes. Detailed sequence analysis of portions of all these cloned genes provides evidence for extensive sequence exchange within the region containing exons 7 and 8 (there are 9 exons altogether) and the intron between them by a gene conversion mechanism. Based on these data and published sequence data for other P-450 isozymes, Dr. Adesnick has proposed gene conversion to be an important evolutionary mechanism for generating genes encoding related cytochrome P-450 isozymes.

The P-450b and P-450e genes are expressed and induced by phenobarbital in the adrenal gland and liver but not in the kidney, brain, or preputial gland (a gland that actively synthesizes steroids). Dr. Adesnick found low levels of expression of the P-450b and/or P-450e genes in the lung. These levels were approximately 10 fold less than the basal level in the liver and were unaffected by phenobarbital administration. A related gene that encodes an as yet unidentified P-450 isozyme was expressed in the preputial gland. The expression of several P-450 genes in organs that are active in steroid synthesis, as well as the known capacity for P-450b and P-450e to metabolize steroids, suggest steroids as the endogenous substrates for the enzymes encoded by this gene subfamily.

Dr. Curtis Omiecinski is investigating the developmental regulation and gene expression of rat liver cytochromes P-450b and P-450e, which are immunologically indistinguishable proteins that are coordinately induced by phenobarbital and exhibit almost identical molecular weights of 52,000 daltons. He has designed and used synthetic DNA oligomer (18'mer) hybridization probes as highly sensitive and discriminatory tools for detecting the individual levels of P-450b and P-450e mRNAs in various tissue samples. It was not previously possible to discriminate between the individual RNAs or genes for P-450b or P-450e, due to their extensive (greater than 97 percent) primary structure homologies. Dr. Omiecinski's hybridization experiments with the oligomer probes demonstrate that, in untreated adult rat liver, very little or no detectable mRNA for either P-450b or P-450e exists; in phenobarbital-pretreated adults, the levels of P-450b and P-450e mRNA increase to approximately 600 and 150 molecules per cell, respectively.

Dr. Omiecinski also used the oligomer probes to obtain a developmental profile of P-450b and P-450e mRNA expression and to determine the responsiveness of these genetic loci to phenobarbital. He found that developing rat embryos <u>in utero</u> are unable to increase the transcription of P-450b and P-450e genes in response to phenobarbital administration. However, almost exactly at the time of birth, the developing rats become genetically responsive to phenobarbital administration. Phenobarbital inducibility of these P-450 mRNAs was maximal in rats at three weeks of age and then decreased moderately to levels of responsiveness that are maintained through adulthood.

Dr. Adesnick has performed immunoscreening of cDNA libraries with antibodies raised against P-450 PB-1 and 2c, which are major constitutive forms in rat liver and has isolated cDNA clones for three distinct but related cytochromes P-450. On the basis of DNA sequence analysis, these cDNA clones encode proteins that are approximately 50 percent homologous to cytochrome P-450b. One of the clones identified with the anti-PB-1 antibody appears to correspond to the mRNA encoding the immunogen, whereas the second appears to correspond to an immunochemically related but heretofore uncharacterized isozyme. Hybridization experiments show that the mRNAs corresponding to one of the clones is induced 8-16 fold by phenobarbital administration, whereas the second is induced only 2-4 fold. Given the fact that these clones represent genes which are in the same family as the P-450b and P-450e genes and that genomic southern blotting with these probes gives non-overlapping patterns which are as complex as those obtained with P-450b or P-450e probes, this gene family appears likely to contain 20 to 30 family members which can be divided into at least two subfamilies.

"Cytochrome P-450 Polymorphism"
R01 GM 31001-03 (Johnson, E.), Scripps Clinic and Research Foundation

Dr. Eric Johnson and his coworkers have characterized variations in the expression of cytochrome P-450 1, which converts progesterone to deoxycorticosterone in the liver. Rabbits exhibit two phenotypes, 21H and 21L, that differ 10-fold in their hepatic progesterone 21-hydroxylase activity. Dr. Johnson's studies, utilizing a highly specific monoclonal antibody to P-450 1, indicate that the microsomal concentration of this P-450 is highly correlated with 21-hydroxylase activity. Differences in the rate of 2-hydroxylation of estradiol and of the conversion of the carcinogen, benzo(a)pyrene, to hydroxylated products are also linked to variations in the hepatic concentration of P-450 1.

Although these differences reflect the microsomal concentration of the enzyme, the 21H and 21L animals contain different electrophoretic variants of P-450 1. These variants were detected for P-450 synthesized in vitro, using mRNA isolated from each phenotype. Breeding studies conducted by Dr. Johnson indicate that the 21H phenotype is inherited in an autosomal dominant manner. The 21L variant seems to be produced only in homozygous recessive inbred strains. Test crosses between 21H and 21L homozygotes exclusively express the electrophoretic variant associated with the 21H parent, with no evidence of expression of the 21L form.

Thus, the electrophoretic variants of P-450 may not be allelic forms. Rather, their expression probably reflects the inheritance of a trans acting regulatory factor. Southern blot analysis using a full-length cDNA encoding a P-450 1 detects multiple gene-like sequences in the rabbit genome. S1 mapping experiments indicate that several homologous mRNAs may be expressed from these genes but that only those highly homologous to P-450 1 are differentially expressed in the 21H and 21L phenotypes.

"Induction and Mode of Action of Hepatic Drug Oxidase"
R01 GM 26114-07 (Schenkman, J.), University of Connecticut Health Center

Dr. John Schenkman's laboratory has identified two additional constitutive forms of cytochrome P-450 in mature rats. Three forms of hepatic microsomal cytochrome P-450, known as RLM2, RLM3, and RLM5, are known to be present in male rats but not

in females. Females have a form designated fRLM4, which is absent in males. Dr. Schenkman isolated two other forms of cytochrome P-450, RLM5a from male rats and fRLM5a from female rats; these two were found to be the same by two-dimensional isoelectric-focusing/SDS-PAGE, peptide mapping and catalytic activity towards drugs and testosterone.

The interactions of cytochrome b_5 with the constitutive forms of cytochrome P-450 of the male rat were compared and were found to differ. Cytochrome b_5 produced only minor shifts in the spin equilibrium of RLM2 and RLM3 but produced large shifts toward the high spin state in RLM5 and RLM5a. The ability of cytochrome b_5 to perturb the spin equilibrium was reflected in its ability to stimulate turnover of the enzyme with drugs and endogenous substrates: no stimulation of aminopyrine, benzphetamine or p-nitroanisole turnover was observed for RLM2 or RLM3, but RLM5 and RLM5a were stimulated 3.5-fold and 14-fold, respectively. Cytochrome b_5 also stimulated testosterone hydroxylation by RLM5 and RLM5a (35 percent and 85 percent, respectively), without altering the isomeric metabolite ratio. The stimulation for testosterone involved V_{max} but not the K_m.

"Prostaglandin 19- and 20-Hydroxylation by Cytochrome P-450"
R01 GM 31296-03 (Masters, B. S.), Medical College of Wisconsin

Prostaglandins are important local mediators of the air passages; they regulate pulmonary smooth muscle contraction and relaxation and blood flow through the pulmonary vasculature by regulating vasoconstriction and vasodilation. Prostaglandin metabolism in the lung undergoes unique alterations during pregnancy as a result of the apparently simultaneous induction of two enzymes that are involved in metabolism of prostaglandins of the E and F series: NAD-dependent 15-hydroxyprostaglandin dehydrogenase and a cytochrome P-450 mediated prostaglandin omega (ω)-hydroxylase. NAD-dependent 15-hydroxyprostaglandin dehydrogenase is a cytosolic enzyme that catalyzes the oxidation of the 15-hydroxyl group that is the initial step in the inactivation of prostaglandin E and prostaglandin F. Cytochrome P-450 mediated prostaglandin ω-hydroxylase catalyzes the hydroxylation of prostaglandins at carbon 20 and represents the initial step in a series of reactions in the formation of dicarboxylic acid derivatives of prostaglandins.

Dr. Bettie Sue Masters purified and characterized the cytochrome P-450 of the prostaglandin ω-hydroxylation system from the lungs of pregnant rabbits. She found that it selectively hydroxylates prostaglandins at carbon atom 20 but does not hydroxylate other substrates of the cytochrome P-450 system, such as drugs or fatty acids. Dr. Masters prepared a polyclonal IgG fraction against this cytochrome and has used it to quantitate the appearance of this novel cytochrome P-450 in lung microsomes during pregnancy. Preliminary immunohistochemical studies identify the Clara cell as a major site of this cytochrome P-450.

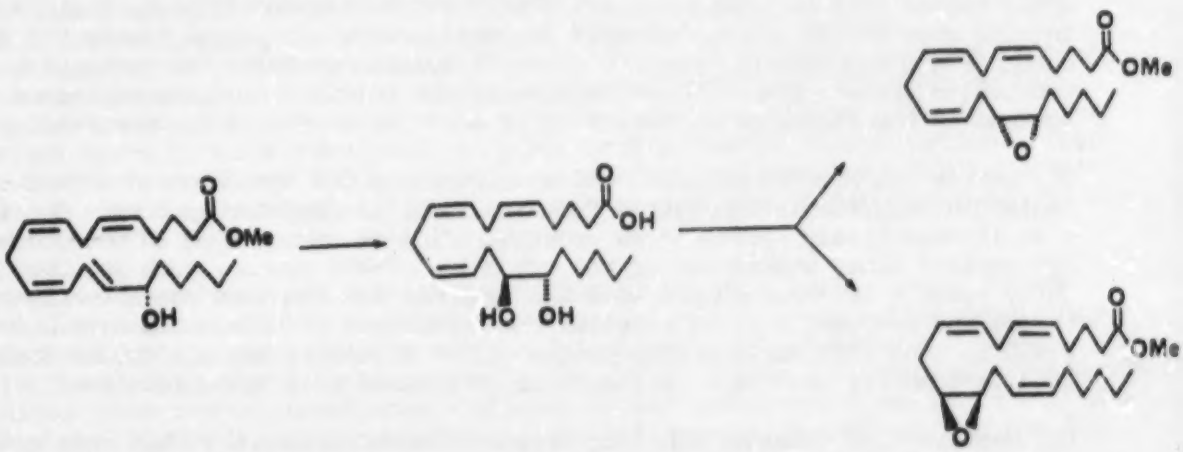
Dr. Masters also obtained RNA from pregnant and nonpregnant rabbit lung and has translated it *in vitro*. Polyadenylated mRNA isolated from pregnant rabbits produces an immunoprecipitable material that cross-reacts with antibody against the cytochrome P-450-mediated prostaglandin ω-hydroxylase, but no such immunoprecipitable material was obtained when polyadenylated mRNA from lungs of nonpregnant rabbits was translated. These experiments are establishing the groundwork for understanding the hormonal changes that regulate the synthesis of cytochrome P-450-mediated prostaglandin ω-hydroxylase during pregnancy.

"Eicosanoid Synthesis"
RO1 GM 31278-02 (Falck, J.), University of Texas Health Science Center, Dallas

Dr. John Falck and his collaborators are examining the involvement of cytochrome P-450 in the production and metabolism of arachidonic acid metabolites. This group showed that the cytochrome P-450 pathway produces 5-,8-,9-,11-,12-, and 15-hydroxyeicosatetraenoic acids (HETEs), ω and ω-1 oxidation products, and four regioisomeric epoxyeicosatrienoic acids. The epoxyeicosatrienoic acids can be further metabolized by cytosolic epoxide hydrolases to vic-diols, which are good substrates for hepatic S-glutathione transferases, affording S-sulfidopeptide adducts analogous to leukotriene C_4.

A novel class of selective arachidonic acid metabolism inhibitors that incorporate an isonitrile functionality has been synthesized. Initial in vitro evaluation showed that they are extremely potent inhibitors of cytochrome P-450 mediated arachidonate metabolism but do not significantly alter metabolism by prostaglandin synthetase purified from ram seminal vesicles, soybean lipoxygenase, or sonicated human platelets. These inhibitors will be powerful tools for studying the physiological role of the cytochrome P-450 pathway in eicosanoid production and will provide a basis for the rational design of more effective therapeutic agents.

Dr. Falck's laboratory also has achieved the first stereocontrolled synthesis of the cytochrome P-450 metabolites 11(S),12(R)- and 14(R),15(S)-epoxyeicosatrienoic acid by exploiting 15(S)-HETE derived precursors (Scheme). In addition, several structural analogs, including 14(S),15(R)-, trans-14(R),15(R)-epoxyeicosatrienoic acid and the corresponding aziridine and episulfide heteroatom derivatives have been prepared, and procedures have been developed for chemically transforming them into glutathione and cysteine adducts.



In vitro biological testing revealed that epoxyeicosatrienoic acids markedly alter sodium and potassium flux and transepithelial voltage in isolated rabbit kidney cortical collecting tubules. Evidence for a contributory role of epoxyeicosatrienoic acids in the release of oxytocin and arginine vasopressin has been obtained in the neural lobe of rat pituitary. These studies are likely to promote a better understanding of polyunsaturated fatty acid metabolism and its relationship to several important biological processes.

"The Induction and Regulation of Hamster Cytochrome P-450"
R01 GM 31584-03 (Chiang, J.), Northeastern Ohio Universities College of Medicine

Dr. John Chiang and his coworkers have focused their research on the induction and regulation of cholesterol 7α-hydroxylase, a cytochrome P-450 dependent monooxygenase that is the first and perhaps most important regulatory enzyme in the bile acid metabolism pathway. A cytochrome P-450 isozyme has been identified and purified from liver microsomes of hamsters pretreated with a diet containing cholestyramine, a bile acid sequestrant. This isozyme has the same apparent molecular weight as the high-spin form (Form 4) isolated from microsomes of 3-methylcholanthrene-treated hamsters. Reconstitution of cholesterol 7α-hydroxylase was achieved by mixing the cytochrome P-450 isozyme, NADPH-cytochrome P-450 reductase and dilauroylglycerol-3-phosphorylcholine. This isozyme is specific in that it catalyzes the 7α-hydroxylation of cholesterol.

Dr. Chiang has examined the effects of phosphatase and kinase on microsomal and reconstituted cholesterol 7α-hydroxylase activities. While alkaline phosphatase inhibits several cytochrome P-450-dependent monooxygenases, such as benzphetamine N-demethylase, 7-ethoxycoumarin O-deethylase and benzo(a)pyrene hydroxylase, cholesterol 7α-hydroxylase was at least 20-100 times more sensitive than the other monooxygenases toward alkaline phosphatase inhibition. Neither cAMP-dependent nor cAMP-independent protein kinase had a significant effect on cholesterol 7α-hydroxylase activity, either in microsomes or in the reconstituted enzyme system. This research should provide information about the regulation of cholesterol 7α-hydroxylase, bile acid metabolism, and cholesterol gallstone formation.

"Selective Destruction of Cytochrome P-450 by Drugs"
R01 GM 25515-07 (Ortiz de Montellano, P.), University of California, San Francisco

Organisms are known to be much more vulnerable to oil-soluble toxins when their P-450 shield is impaired, but little information is available on whether the loss of P-450 itself, in the absence of a toxic challenge, is deleterious. To answer this question, Dr. Paul Ortiz de Montellano is seeking to eliminate the P-450 enzymes involved in drug metabolism without eliminating those P-450 enzymes that are an integral part of essential metabolic processes, such as sterol biosynthesis. This need is met by 1-aminobenzotriazole, a suicide substrate developed in the laboratory of Dr. Ortiz de Montellano that irreversibly inactivates up to 80 or 90 percent of the hepatic cytochrome P-450 in uninduced rats without detectably affecting any of the sterol biosynthetic enzymes so far tested. It is therefore significant that no detectable changes in a variety of parameters that measure toxicity or in the apparent health of the animals result from administration of the agent to rats for up to two weeks. Thus, drug metabolizing forms of cytochrome P-450 appear to be important primarily because of the protection they provide against xenobiotics. 1-Aminobenzotriazole should find wide utility in studies of the role of cytochrome P-450 enzymes in physiological and toxicological processes.

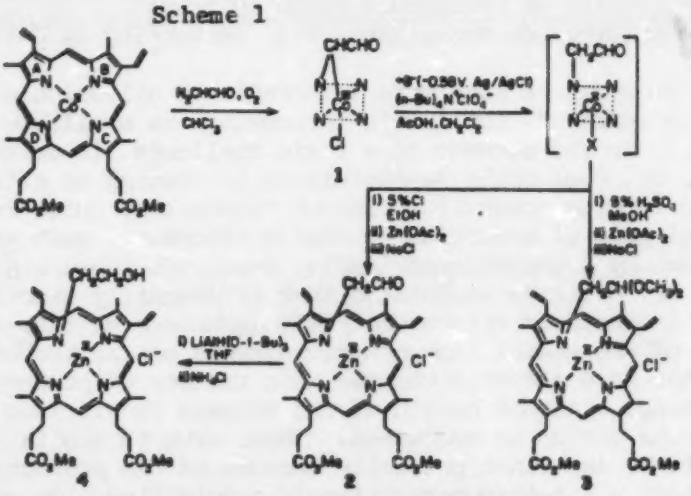
In order to examine more precisely the metabolic role of a single form of cytochrome P-450, Dr. Ortiz de Montellano has been attempting to differentially inactivate the closely related enzymes that hydroxylate fatty acids, prostaglandins, and leukotrienes. He has found that the incorporation of an acetylenic group into the substrates of these different enzymes results in their highly specific, differential inactivation. Thus, the enzyme that hydroxylates the terminal carbon of

short chain fatty acids (lauric acid) can be inactivated by 10-undecynoic acid without significantly inactivating the enzymes that hydroxylate prostaglandins and leukotrienes, whereas 17-octadecynoic acid, the longer chain analog, inactivates the latter enzymes without affecting the former. These inhibitors and closely related analogs are being utilized to elucidate the role of fatty acid hydroxylases in a variety of physiological processes. The demonstration that closely related cytochrome P-450 enzymes can be differentially inactivated lends support to the feasibility of developing inhibitors of specific cytochrome P-450 enzymes that could be used as therapeutic or insecticidal agents.

"Myeloperoxidase, Sirocheme, B12: Synthesis and Function"
R01 GM 29198-04 (Dolphin, D.), University of British Columbia

Dr. David Dolphin is using N-alkylprotoporphyrin IX dimethyl esters as probes for the action of cytochromes P-450. These substrates lead to suicidal N-alkylation of the prosthetic heme and subsequent inhibition of ferrochelatase. He synthesized N-formylmethylprotoporphyrin IX dimethyl ester zinc chloride, 2, and the corresponding dimethyl acetal 3 by the electrochemical reduction of (N,Co-formylmethylene) protoporphyrin IX dimethyl ester cobalt(III) chloride, 1. Species 1, in which the cobalt and a porphyrin nitrogen are bridged by a single carbon atom, was prepared by reacting protoporphyrin IX dimethyl ester cobalt(II) with diazoacetaldehyde. N-(2-Hydroxyethyl)-protoporphyrin IX dimethyl ester zinc chloride, 4, was obtained by reducing the corresponding N-formylmethyl complex with $LiAlH(O\text{-}\underline{tert}\text{-}C_4H_9)_3$. The free bases of all of the zinc complexes were obtained by acid-catalyzed demetallation. These reactions are outlined in Scheme 1.

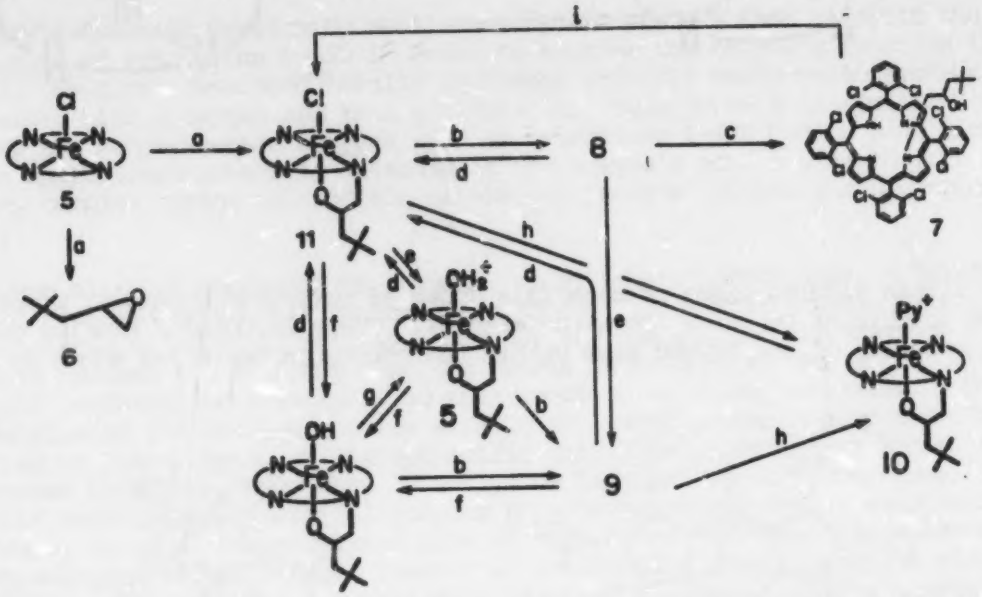
Scheme 1



[One of the four possible isomers is shown for 2, 3 and 4]

Dr. Dolphin finds 5,10,15,20-Tetrakis(2,6-dichlorophenyl)porphinatoiron(III), 5, to be an effective catalyst for the hydroxylation of alkanes and the epoxidation of olefins, using the oxidant iodosopentafluorobenzene. When 4,4-dimethyl-1-pentene is the substrate, the principal product is the corresponding epoxide 6. Suicide inhibition paralleling the in vivo destruction

of cytochrome P-450 occurs to give an N-alkylporphyrin, 21-(4,4-dimethyl-2-hydroxypentyl)-5,10,15,20-tetrakis(2,6-dichlorophenyl)porphine, 7. These reactions are outlined in Scheme 2.



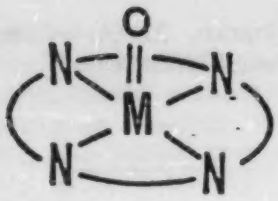
Scheme 2 Interconversions of the N-alkylporphyrin and its iron complexes

a) 4,4-dimethyl-1-pentene, F_5C_6I0/CH_2Cl_2
b) F_5C_6I0/CH_2Cl_2
c) c.HCl/AcOH
d) 1% aq.HCl/CH_2Cl_2
e) H_2O/CH_2Cl2
f) aq.$NaHCO_3/CH_2Cl_2$
g) $AcOH/CH_2Cl_2$
h) Pyridine/CH_2Cl_2
i) $FeCl_2THF$,

Hence, this model system seems to mimic the suicide inhibitors characteristic of microsomal P-450 and may be useful for further research the chemistry of these enzymic systems.

"Iron Porphyrin Activation of Dioxygen/Small Molecules"
R01 GM 26226-07 (Balch, A.), University of California, Davis

The structures 1 and 2 shown below (with M = iron) have been proposed for the highly oxidized forms of heme proteins involved in the enzymatic action of the peroxidases and cytochrome P-450 and in the self-destruction of heme (heme catabolism). Structure 1 involves a classical metal oxo unit and is a well-established structural unit for a variety of metal ions, including iron(IV). Structure 2 is a non-classical structure in which an oxygen atom has become inserted into N-Fe bond. Although theoretical calculations have suggested that 2 may be more stable than 1, particularly for metal ions other than iron, no examples of structure 2 have been unambiguously characterized until this past year. Dr. Alan Balch's research group undertook the preparation of examples of structure 2 by way of



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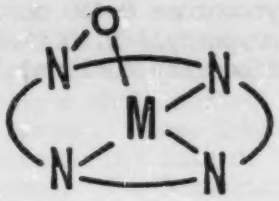

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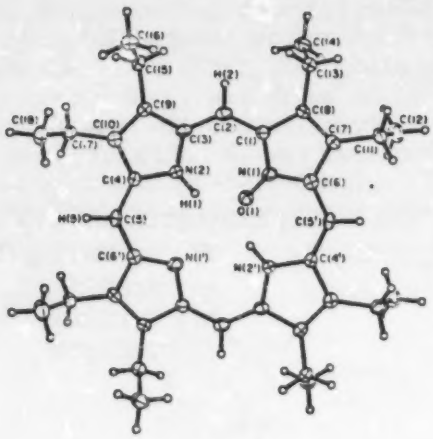
the little studied class of materials known as porphyrin N-oxides, prepared from free porphyrins by oxidation with peracids. These structural studies confirm the presence of one oxygen atom within the porphyrin center as shown in 3, a



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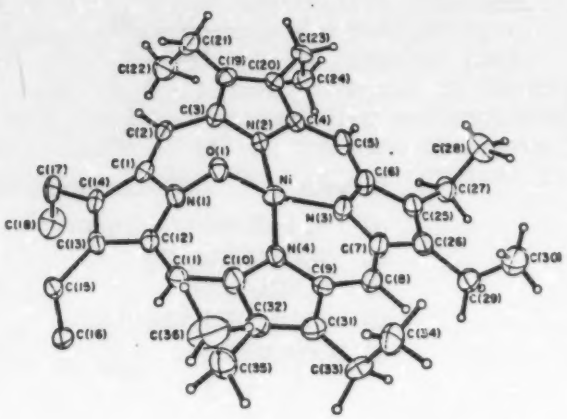

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drawing derived from X-ray diffraction analysis. Professor Balch, Dr. M. Olmstead, and Dr. W. Y. Chan find that the addition of metal ions to these porphyrin N-oxides produces the desired metal complexes. Especially mild procedures for introducing the metal ion into the center of the porphyrin are required; otherwise, the oxygen atom is displaced to carry out oxidation reactions. Nickel and copper complexes analogous to structure 2 have been prepared; a drawing of the nickel complex derived from the X-ray structural study is shown in 4.

These models are important for several reasons. They confirm that structural type 2 can be a stable entity and give precise geometric details about this structure. They offer well-defined examples against which information derived from enzymatic intermediates can be compared. They allow one to test the ability of this structural type to act as an oxidizing agent, and they give the opportunity to study the migration of an oxygen atom from the metal out to the edge of the porphyrin, where porphyrin destruction is known to occur during heme catabolism.

"Models of Cytochrome P-450: Advances in Mimicking the Catalytically Active Ternary Complex"
R01 GM 10040-22 (Busch, D.), Ohio State University

Dr. Daryle Busch is developing metal-macrocyclic systems that resemble the catalytic site of cytochrome P-450 in order to examine the enzyme's function in greater detail. He first developed totally synthetic iron(II) macrocyclic systems that reversibly bind dioxygen and then activate it. This process is similar to that used by the monooxygenase enzymes such as cytochrome P-450, which bind dioxygen and an appropriate substrate molecule at the enzyme's active site to form a ternary complex, prior to reaction of the two "guests" within the hydrophobic cavity.

Dr. Busch also has succeeded in forming, under ambient conditions, a binary complex composed of host (a paramagnetic copper(II) lacunar complex) and guest (an aliphatic or aromatic hydroxy compound) and has studied the complex by nuclear magnetic resonance. He found that the guests interact with the cavity in specific preferred orientations, so that reaction of these compounds in the active site of the model complexes with an activated oxygen moiety might be expected to yield regio- or stereospecific oxidation products, as occurs for cytochrome P-450 enzymes. Most recently, he has used ternary complexes, composed of macrocyclic cobalt(II) complex plus bound dioxygen plus associated substrate, to model the catalytic site of cytochrome P-450 and has observed the same phenomenon of hydrophobic guest-host interaction when the active site of the complex also contains a potentially reactive dioxygen. This suggests the feasibility of combining results on the guest-host complexation with those on metal-ion activation of dioxygen to provide a family of complexes that are viable working models for monooxygenase enzymes such as the cytochromes P-450.

Metallochemistry

"Studies on Superoxide Radicals and Metal-Oxy Complexes"
R01 GM 23656-08 (Bielski, B.), Brookhaven National Laboratory

The superoxide radical (O_2^-) is believed to be involved in oxygen toxicity in biological systems. Research on its reactivity has resulted in the opinion that O_2^- is not itself reactive and, therefore, not directly responsible for the deleterious effects observed in its presence. This led to the consideration of other O_2^--derived active oxygen species, such as hydroxyl radicals or singlet oxygen, as the true damaging species.

Dr. Benon Bielski and his colleagues are studying the reactivity of HO_2/O_2^- ($HO_2 \rightleftharpoons H^+ + O_2^-$, pK = 4.8) with biologically important compounds in the absence of contaminants such as metals, that could produce these active oxygen species. They showed previously that O_2^- does not react with polyunsaturated fatty acids but that its acid form, the perhydroxyl radical (HO_2), reacts with these acids at significant rates ($k \sim (1\text{-}5) \times 10^3\ M^{-1}\ sec^{-1}$). The low reactivity of O_2^- may enable it to diffuse significant distances and thus reach vital targets inaccessible to more reactive species. At physiological pH, less that 0.1 percent of O_2^- is present as HO_2. However, the pH at membrane surfaces is known to be lower by several units and, therefore, most of the unreactive O_2^- could be converted

into the more reactive HO_2. Reaction of HO_2 with the lipid domain of the membrane could then initiate a chain oxidation, leading to impairment of the cell's function.

In view of the ubiquity of metals in living cells, the role of metals in the deleterious effects observed in systems containing HO_2/O_2^- is of great interest. Until now, studies related to these systems have been carried out at low pH, and not in the biological pH range. Recently, Dr. Bielski has employed pulse-radiolysis to study reactions of metal complexes and metal-oxy complexes at pH 5-7. He finds that MnO_2^+/Mn^{3+} react with compounds such as ascorbic acid (vitamin C), trolox (a synthetic vitamin E analog), and the coenzymes NADH/NADPH at fast rates ($k = 10^5$-10^6 M^{-1} sec^{-1}). In fact, recent studies involving Fe^{3+} and ascorbic acid show that at pH 7 the reaction occurs at 5×10^6 M^{-1} sec^{-1}.

"Reactions and Mechanisms of Metallosulfur Complexes"
R01 GM 25122-08 (Rakowski-DuBois, M.), University of Colorado

Dr. Mary Rakowski-DuBois has synthesized isostructural dimeric molybdenum complexes of the formula $[(C_5H_5Mo)_2S_2CH_2(\mu\text{-}S)(\mu\text{-}SCH_3)]^n$ (n= +1, 0, -1) and has characterized the reactivity of their bridging sulfido ligands with electrophiles and nucleophiles as a function of the dimer's overall charge. The cationic dimer (n = +1) reacts with molecular hydrogen under mild conditions (25°C, 1 atm hydrogen) to form the neutral hydrosulfido bridged dimer $(C_5H_5Mo)_2S_2CH_2(\mu\text{-}SH)(\mu\text{-}SCH_3)$. The reactant $[(C_5H_5Mo)_2S_2CH_2(\mu\text{-}S)(\mu\text{-}SCH_3)]+$, which is a diamagnetic Mo(IV)/Mo(IV) dimer, does not react detectably with hydrogen in chloroform solution until an equivalent of base, such as triethylamine, is added. The production of the neutral hydrosulfido complex is accompanied by formation of the protonated base. The products are consistent with a heterolytic cleavage of hydrogen in the overall process. These reactions may aid understanding of the mode of reactivity of the hydrogenase enzymes, which are known to contain sulfur-ligated metal centers and which catalyze the interconversion of protons and hydrogen in the presence of an electron carrier.

"The Function of Molybdenum in Biological Systems"
R01 GM 08347-24 (Spence J.), Utah State University

The molybdenum centers of several enzymes (for example, xanthine oxidase, sulfite oxidase, nitrate reductase) undergo rapid one-electron redox reactions during catalysis, with the metal cycling between the +6, +5 and +4 oxidation states. However, few isolated dioxo-Mo(VI) complexes are reducible to monomeric Mo(V) and Mo(IV) species because of the formation of biologically irrelevant oxo-bridged (Mo(V) dimers:

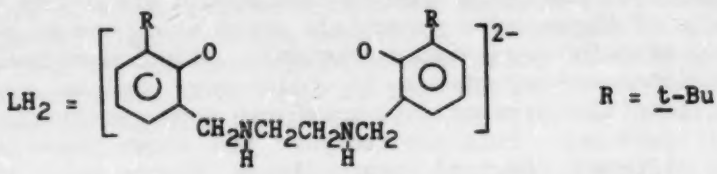
$$2\ Mo(VI)O_2L_n \xrightarrow{2e^-} Mo(V)_2O_3L_{2n}$$

(L = ligand(s))

Recently, Dr. Jack Spence synthesized a number of new dioxo-Mo(VI) complexes which, upon reduction, give monomeric Mo(V) and Mo(IV) complexes. He accomplished this by incorporating sterically bulky groups into the ligand that prevent the close approach required for oxo-bridge formation. These complexes mimic the enzyme molybdenum centers, in which dimer formation is prevented by protein constraints. If

two secondary amino groups are present in the Mo(V) monomer, an oxo is lost and the amino groups deprotonated, giving H_2O and the Mo(V)-diamido complex:

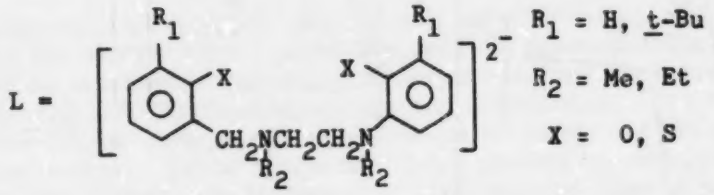
$$MoO_2LH_2 \xrightarrow{e^-} MoOL^- + H_2O$$



$$LH_2 = [SC(Et)_2CH_2NCH_2CH_2NCH_2C(Et)_2S]^{2-}$$

If the amino groups of the Mo(V) monomer are tertiary, then stable dioxo-Mo(V) complexes are formed in situ. These in turn can be protonated to give Mo(V)O(OH)L species, or they can be substituted with F^- or other anions to give MoO(V) (X) L complexes, again mimicking the behavior of the molybdenum centers of enzymes:

$$MoO_2L \xrightarrow{e^-} MoO_2L^- \xrightarrow{H^+} MoO(OH)L \xrightarrow{F^-} MoO(F)L$$



Dr. Spence has obtained EPR coupling constants for ^1H, ^{17}O, and ^{19}F for these MO(V) complexes and is comparing them with the corresponding parameters for the enzyme Mo(V). These data, combined with X-ray structural results for the complexes, should provide information concerning structures of the active site centers of molybdenum-containing enzymes.

"Biologically Relevant Kinetics of Iron Hydroporphyrins"
R01 GM 31554-02 (Strauss, S.), Colorado State University
"Molecular Probes of the Mechanism of Cytochrome P-450"
R01 GM 25923-07 (Groves, J.), University of Michigan, Ann Arbor

Dr. Steven Strauss is investigating the thermodynamic, kinetic, structural, and magnetic properties of iron porphyrins and hydroporphyrins. He has found that porphyrins and chlorins differ by as much as two orders of magnitude in their ligand binding to form high-spin 6-coordinate complexes. Chlorin complexes have the larger binding constants because of their larger cores, which enables them to accommodate high-spin complexes more readily than can porphyrins. Dr. Strauss also has investigated the interaction of the metal with an axial ligand in two

series of porphyrin and hydroporphyrin iron(III) μ-oxo dimers. He found that ferric complexes form weaker bonds to the μ-oxo ligand. Crystal structure analysis of $[Fe(TPC)]_2O$ revealed that the average Fe-N and Fe-O distances are longer for the TPC complex than for $[Fe(TPP)]_2O$. In addition, Dr. Strauss finds vastly different magnetic properties (and different Mossbauer spectra) between iron porphyrins and chlorins. Dr. John Groves has developed techniques for the manipulation of oxoiron(IV) porphyrin species that should yield data on their redox potentials. Knowledge of these redox potentials would allow one to predict when outer sphere electron transfer is a viable substrate oxidation mechanism and when covalent intermediates are expected to be obligatory. Initial results with chloro- and hydroxy-iron(III) tetramesityl porphyrin show two clearly reversible oxidation waves (Figure 1a). Bulk electrolysis just above these two waves produces two distinctly different spectral intermediates (Figure 1b). The first of these (B) is certainly the iron(III) porphyrin radical. The identity of the second (C) is uncertain but may be an iron(IV) species or a porphyrin dication.

Studies such as those of Drs. Grove and Strauss should shed light on the structure and function of enzymes that contain porphyrins or chlorin, such as E. coli sulfite reductase and various nitrite reductases, which have been shown to contain an iron isobacteriochlorin magnetically coupled to an Fe_4S_4 cluster.

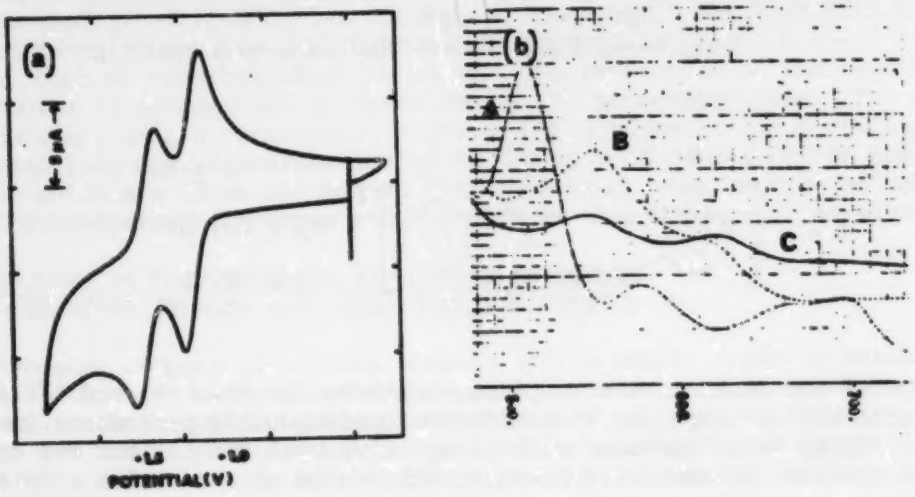


Figure 1

"Mitochondrial Iron-Sulfur Proteins"
R01 GM 33806-01 (Johnson, M.), Louisiana State University

Iron-sulfur clusters greatly outnumber cytochromes in the mitochondrial respiratory chain, but their function and diversity is poorly understood. Dr. Michael Johnson has utilized a novel combination of low temperature magnetic circular dichroism and electron paramagnetic resonance spectroscopy to study iron-sulfur clusters in beef heart mitochondrial enzymes. He has identified three distinct clusters in succinate dehydrogenase: S1, $[2Fe-2S]^{1+,2+}$; S2, $[4Fe-4S]^{1+,2+}$; S3, a

[3Fe-xS] cluster similar to those recently identified in bacterial ferredoxins and aconitase. Parallel studies of fumarate reductase from E. coli, an enzyme that catalyses the reverse reaction, reveal an analogous cluster and subunit composition. Morever, electron paramagnetic resonance studies of bacterial whole cells show the [3Fe-xS] center to be present in vivo, indicating that it is unlikely to be an isolation artifact in either enzyme. This finding is important, because a similar type of cluster in aconitase was shown to be produced by oxidative damage of a [4Fe-4S] cluster during isolation. Comparison of the published amino acid sequence data for the bacterial fumarate reductase with the cluster composition established in this work suggests that all three iron-sulfur clusters are located in the smaller subunit (M_r 27,000) rather than the larger, flavoprotein subunit (M_r 66,000).

Since both the binuclear and trinuclear iron-sulfur clusters are reducible by succinate within the catalytic turnover time, they presumably function as electron carriers. A functional role for the tetranuclear cluster, which has a midpoint potential far below that of the succinate/fumarate couple, has yet to be established. Continuation of these studies should provide information about the molecular mechanism of transferring electrons from succinate to ubiquinone in the mammalian respiratory chain.

Oxidation/Reduction Reactions

"Reversible Redox Control of Macrocycle-Cation Binding"
RO1 GM 33940-01 (Echegoyen, L.), University of Miami

The ability to bind cations such as sodium, potassium, and calcium and to regulate their concentrations inside the cell is essential for proper physiology in biological systems. Selective transport across membranes is a major process for regulating cation concentrations and activities. Dr. Luis Echegoyen has been using "switchable" macrocyclic polyethers to study cation exchange and transport through membranes. These compounds can form strong complexes with a variety of cations under certain conditions but bind cations less strongly under other conditions, thereby effecting cation release. Dr. Echegoyen's approach has been to attach to the macrocyclic polyether a chemical group capable of being reduced to an anionic form by electron transfer. The resulting species thus contains a negative charge in the sidearm substituent which, if appropriately situated, can interact strongly with a bound cation, thereby enhancing the cation binding ability of the polyether. Removing the electron causes the system to revert to its low binding form and to release the cation. This electrochemical approach provides the necessary control to effect cation binding enhancement.

Dr. Echegoyen has synthesized molecules containing 4, 5, and 6 oxygens in the polyether macroring. Nitrobenzene and anthraquinone sidearms have been placed on these ring systems and their binding properties investigated using cyclic voltammetric techniques and Electron Spin Resonance spectroscopy (ESR). Results show binding enhancements in the order of 10^3-10^6 for alkali metal cations. These enhancements, when coupled to the base binding abilities of the neutral compounds, result in binding constants approaching those of cryptands. For these modified macrocyclic ethers, enhancement factors are always in the order of Li > Na > K. A further modification has been the synthesis of a bicyclic version containing

the azobenzene group. This latter system exhibits selective binding enhancement for K but not for either Li or Na.

"Oxidation Chemistry of Indoles"
R01 GM 32367-02 (Dryhurst, G.), University of Oklahoma

A number of indolic compounds are of fundamental importance in both the animal and plant kingdoms. Much of the chemistry of such indoles in living organisms seems to involve rather complex oxidation reactions, the mechanisms or products of which are not well understood. Dr. Glenn Dryhurst and his colleagues are studying the fundamental oxidation chemistry of biologically significant indoles, using primarily electrochemical and related analytical methodologies. One of the first indoles so studied is the chemical neurotransmitter 5-hydroxytryptamine. The electrochemical oxidation of 5-hydroxytryptamine is exceedingly complex; one minor route leads to the powerful and selective neurotoxin 5,7-dihydroxytryptamine, which is rapidly oxidized further to 4,5,7-trihydroxytryptamine and finally to 5-hydroxytryptamine-4,7-dione. The latter compound is a major electrochemical and autoxidation product of 5-7-dihydroxytryptamine. Preliminary studies using mice indicate that 5-hydroxytryptamine-4,7-dione is a more potent central nervous system toxin than 5,7-dihydroxytryptamine. These results demonstrate for the first time a chemical pathway from 5-hydroxytryptamine to 5,7-dihydroxytryptamine and suggest possible minor oxidative metabolic pathways from the neurotransmitter to at least two powerful neurotoxins.

The electrochemical oxidation of L-tryptophan at graphite electrodes in aqueous solution proceeds by an initial, irreversible two-electron reaction, giving a very reactive 3-methyleneimine intermediate. This intermediate is extremely electrophilic and is attacked by nucleophiles present in the reaction solution. Nucleophilic attack by water leads to two more intermediates which can be detected by thin-layer spectroelectrochemistry and cyclic voltammetry. Further chemical and/or electrochemical reactions of these intermediates lead to the formation of two stereoisomers of 2-carboxy-3a-hydroxy-1,2,3,3a,8,8a-hexahydropyrrolo-(2,3b)-indole, oxindolylalanine, dioxindolylalanine and kynurenine. In addition, at least two oligomeric and/or polymeric products are formed. Between pH 4 and 7, horseradish peroxidase catalyzes the oxidation of L-tryptophan to many of the same products. Thus, the chemical pathways followed in both the electrochemical and enzymatic reactions appear to be very similar, if not identical. Such information could provide guidance to understanding mental illnesses related to anomalous metabolism of 5-hydroxytryptamine.

"Leukocyte, Enzyme and Drug Chemiluminescence Studies"
R01 GM 32974-02 (Kanofsky, J.), Veterans Administration Hospital, Hines, IL

Singlet oxygen is a short-lived, highly toxic species which may be important in a number of processes of medical interest. Studies in Dr. Jeffrey Kanofsky's laboratory have been directed at the identification of biochemical sources of singlet oxygen, using its chemiluminescence at 1268nm. These investigations have been made possible by the development of spectrometers that are highly sensitive at this wavelength. Dr. Kanofsky and associates have found that lipoxygenase-catalyzed oxidations are accompanied by emission at 1268 nm. Using three soybean lipoxygenase isozymes as models of corresponding mammalian enzymes, Dr. Kanofsky found the biochemical requirements for singlet oxygen production to be similar to those for co-oxidation. These are summarized below:

Isozyme	pH Optimum	Co-oxidation	Singlet Oxygen Production
L1	9.0	moderate	moderate
L2	6.5	very low	very low
L3	6.5	high, if hydroperoxide present	high, if hydroperoxide present

Dr. Kanofsky's results support the hypothesis that singlet oxygen may be formed during lipoxygenase-catalyzed oxidation.

"Singlet Oxygen in Biological Environments"
R01 GM 24235-07 (Rodgers, M.), University of Texas, Austin

Photodynamic effects in biological systems include health problems such as porphyria, sunburn and skin cancer; a beneficial aspect is the use of photodynamic damage in tumor therapy. Tumor phototherapy makes use of the fact that photoreactive pigments such as porphyrins localize preferentially in tumor tissue. Upon irradiation with red light, the tumor-bound porphyrins generate singlet oxygen within the cellular environment. This chemically reactive species is thought to initiate tissue-damaging processes, leading to eventual cell death and consequent tumor regression.

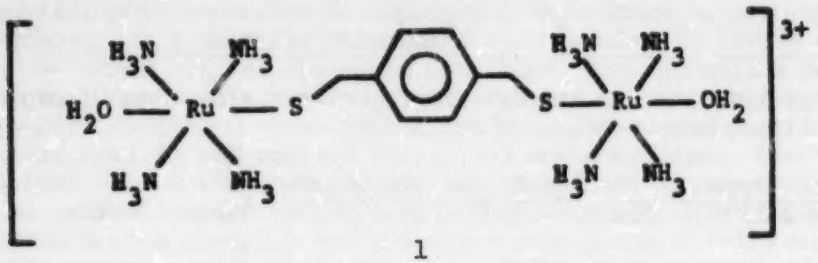
Study of the natural decay time of singlet oxygen in various organic solvent systems led Dr. Michael Rodgers to realize that the weak but significant solvent-derived deactivation component is an additive property. That is, contributions to the deactivation rate by individual sub-molecular residues (methyl, methylene, methine, hydroxy, nitrile, etc.) are constant, and that the total molecular deactivation rate can be computed by adding together the contributions of each grouping. This completely new contribution to the area of singlet-oxygen chemistry has far-reaching fundamental significance. Dr. Rodgers' finding was made possible by the development in his laboratory of an infra-red luminescence apparatus for the direct observation of the very weak infra-red light emission from the singlet-oxygen molecule. This apparatus has a time resolution of better than one microsecond--a timescale not yet achieved by any other laboratory.

Dr. Rodgers has used this instrument to look for singlet oxygen production in resealed erythrocyte cell ghosts that contain added porphyrin and have been irradiated with green light from a high power laser. Not only was it possible to detect singlet oxygen luminescence from such systems, but also to quantify its concentration and decay time. Thus, it may be possible to detect singlet-oxygen production in actual cellular systems.

"Through-Space Electron Transfer Reactions"
R01 GM 31383-03 (Lewis, N.), University of New Brunswick

For more than a decade, researchers have speculated that sulfur atoms and benzene rings play an important role in metalloproteins that have redox functions. Based on this, Dr. Nita Lewis has prepared models which are designed to incorporate molecular fragments found along the presumed electronic pathways in proteins. For example, in compound 1, the sulfur atoms and benzene rings are placed in a

rigid environment and are situated close enough to each other so that their orbitals can overlap. There are two ways that an electron can pass through a molecule like this. The first is a "through-bond" transfer, in which the electron is presumed to follow the molecular framework. This is prevented in compound 1



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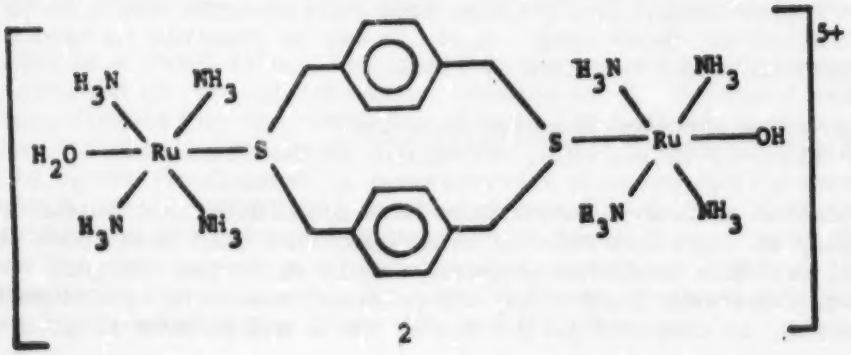
by the two methylene groups, which serve as insulators. It has never been conclusively proven that an electron can pass through a fully saturated carbon like the two methylenes, in an unstrained environment, at any measurable rate. Thus, the electron probably is forced to use the only other possible mechanism available to it, that is the "through-space" mechanism. "Through-space" transfer is so named because the orbitals used in transferring the electron belong to atoms that are not connected directly to each other. This is thought to be the type of transfer that occurs in redox proteins.

In compound 1, the electron is thought to pass directly from a lone pair orbital on the sulfur atom to the p-orbitals of the benzene ring. It then probably passes along to the lone pair orbital on the second sulfur atom and finally exits the bridging ligand by entering an orbital on the metal atom. However, the intervalence band corresponding to a through-space transfer is ten times narrower than predicted by theory. This is tentatively attributed to coupling of this transition to a vibrational mode of the ligand. The electrochemistry (E_f = 0.27 and 0.37 V. versus a standard calomel electrode) indicates that the metal centers are strongly coupled electronically.

Dr. Lewis also has developed the model system shown below, which has two benzene rings sitting in a face-to-face orientation.



2

This molecule apparently shows simultaneous two-electron transfers on the cyclic voltammetric time-scale at 0.11 and 0.28 V. (versus a standard calomel electrode), taking each ruthenium formally from Ru(II) to Ru(IV). Such behavior is unprecedented and may indicate that certain metalloproteins having this feature could function as two-electron redox systems.

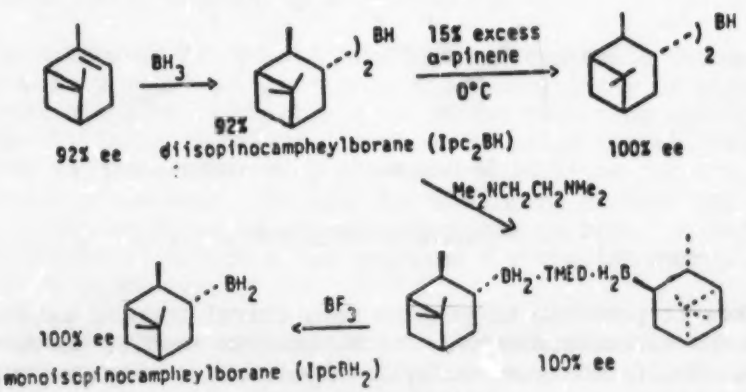
Asymmetric Synthesis

"Asymmetric Synthesis via Chiral Hydroboration"
R01 GM 10937-22 (Brown, H.), Purdue University

Dr. Herbert Brown and his coworkers recently discovered a general method of asymmetric synthesis for preparing, in optically pure form, almost any chiral compound. The method involves hydroboration, which Dr. Brown discovered and developed and for which he was awarded the Nobel prize for chemistry in 1979.

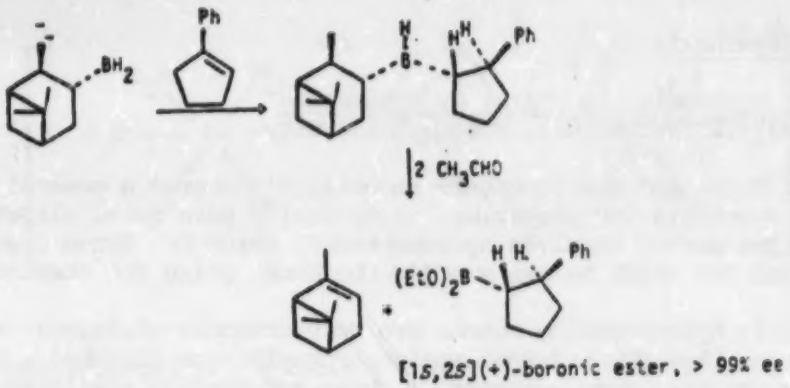
Two asymmetric hydroborating agents are very commonly utilized: diisopinocampheylborane (Ipc_2BH) and monoisopinocampheylborane ($IpcBH_2$). Both reagents are readily obtained from α-pinene, a cheap and readily available material prepared from oil of turpentine. Commercial α-pinene is only 92 percent optically pure. However, if the crystalline Ipc_2BH formed upon hydroboration of α-pinene (92 percent enantiomeric excess) is allowed to stand at 0°C in the presence of a slight excess of α-pinene, the major isomer becomes incorporated into the crystalline reagent, leaving the unwanted minor isomer in solution.

Because the reaction commonly proceeds rapidly to the dialkylborane stage, the monoalkylborane must be prepared by an indirect route. Treatment of Ipc_2BH (92 percent enantiomeric excess) with one-half molar equivalent of N,N,N',N'-tetramethylethylenediamine (TMED) provides the 2:1 adduct, $TMED\text{-}2BH_2Ipc$, with liberation of one molar equivalent of α-pinene. This adduct crystallizes out in enantiomerically pure form. The pure reagent, $IpcBH_2$, is readily liberated by treating the adduct with boron trifluoride:



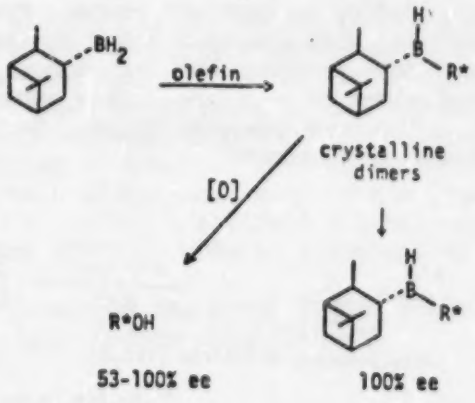
Preparation of Ipc_2BH and $IpcBH_2$

Three recent developments in Dr. Brown's laboratory are of major importance. First is the discovery that treating the asymmetric hydroboration products with acetaldehyde removes the isopinocampheyl group as α-pinene, yielding the optically active boronic esters.



Synthesis of Optically Pure Boronic Esters

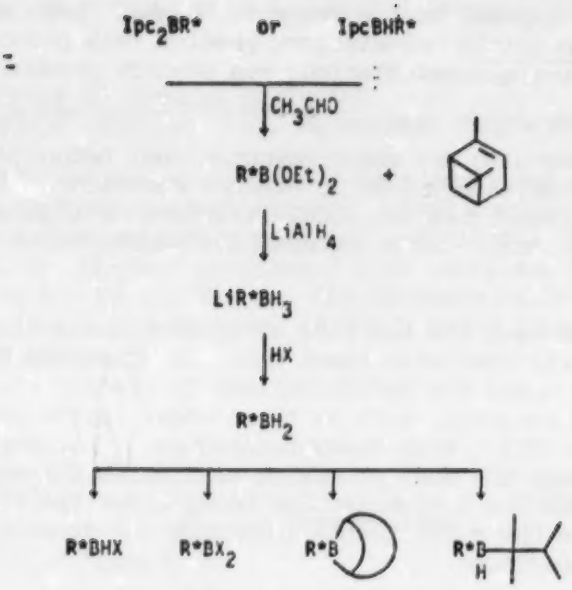
Second is the finding that the reaction products from $IpcBH_2$ and hindered prochiral alkenes, IpcR*BH, exist as crystalline dimers. Simple crystallization provides a product of essentially 100 percent optical purity.



Preparation of IpcR*BH of Essentially 100% ee

This makes it possible to prepare many chiral boronic esters of essentially 100 percent enantiomeric excess. Third, one can readily convert these boronic esters into the corresponding monoalkylborohydrides and, by treatment with acid, obtain the optically active monoalkylborane. The optically active borane can be converted by known methods into all of the organoborane reagents which have been found to be valuable in syntheses.

The whole process is summarized below.



Synthesis of Boron Intermediates of 100% ee

It is well established that alkyl groups can be transferred from boron to essentially any element of interest, including carbon, with complete retention of stereochemistry. Thus, it is now possible to plan a rational synthesis of practically any compound containing an asymmetric center, either the (+) or (-) isomer, in essentially 100 percent optical purity.

"Predicting Stereoselectivities of Synthetic Reactions"
R01 GM 30293-04 (Houk, K.), University of Pittsburgh

In each step in the synthesis of natural products and synthetic therapeutic drugs, it generally is necessary to control not only the connectivity of atoms, but also the three-dimensional shape or stereochemistry of the resulting molecule. Dr. Kendall Houk is utilizing theoretical methods to probe the electronic origins of stereoselectivity in order to develop practical guidelines for the design of useful stereoselective processses. He also is developing methods for the quantitative prediction of the products of organic reactions in order to reduce the trial-and-error experimental effort often required for the laboratory demonstration of new stereoselective processes.

Dr. Houk has made significant advances in the basic understanding of stereospecific additions of various reagents across the carbon-carbon double bonds of chiral allylic alcohols and ethers. These reactions are of considerable interest to synthetic chemists since the products contain the functionality necessary for synthesizing a variety of natural products, especially the polyether antibiotics. Theoretical calculations and experimental studies in Dr. Houk's laboratories showed that in electrophilic additions, allylic ethers prefer to react in such a way that the allylic CO bond remains close to the plane of the alkene double bond. When additional groups are attached to the chiral center, this "anchoring"

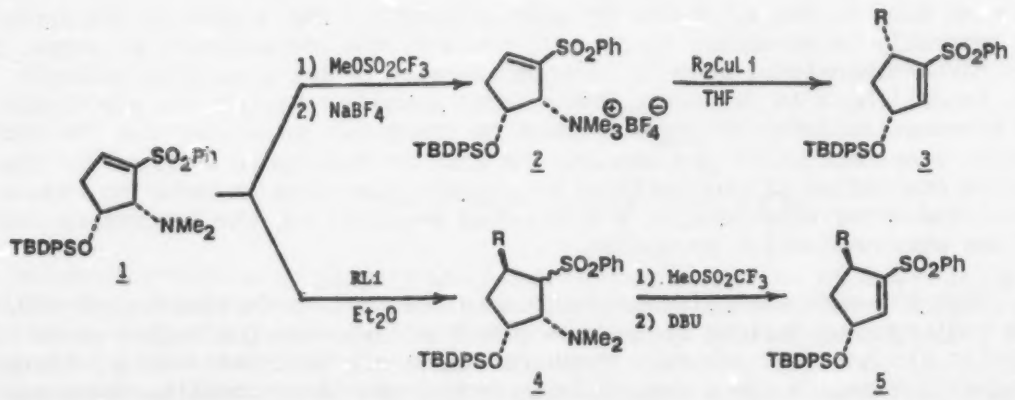
of the allylic oxygen orients the additional groups so that the two sides of the alkene are differentially exposed to the reagent. Highly stereoselective attack is frequently favored. Ab initio calculations predict this phenomenon for simple cases, and empirical force-field calculations can predict product ratios for more complex cases.

Dr. Houk and his colleagues also are using computational techniques to directly locate and examine the transition states of organic reactions. So far, they have located transition state geometries for aldol reactions of lithium and boron enolates, Diels-Alder reactions, and organometallic additions to alkenes and carbonyls.

Still another approach has been the modeling of synthetic reactions by computer in order to study asymmetric synthetic reactions. For example, Dr. Houk and others have used the force-field model for hydroborations to predict the stereoselectivities of new hydroborating reagents, such as those under investigation in Professor Masamune's laboratories at MIT. With these techniques, a variety of structures can be surveyed to determine the most promising candidates for experimental study. A force-field model for intramolecular Diels-Alder reactions has also been devised that explains how substituents influence the stereoselectivities of synthetically useful reactions.

"Stereoselective, Chemodirected Formal S_N2' Addition of Organometallic Reagents to Beta'-Amino Cyclopentenyl Sulfone Derivatives"
R01 GM 32693-02 (Fuchs, P.), Purdue University

Dr. Philip Fuchs has developed a highly efficient and almost totally stereoselective method for synthesizing functionalized cyclopentenyl sulfone derivatives. This method involves the chemospecific generation of either stereoisomer 3 or 5, starting with amine 1. As shown in the scheme, treatment of amine 1 with methyl trifluoromethanesulfonate, followed by counterion exchange with sodium tetrafluoroborate, gave ammonium salt 2, which upon treatment with a number of homocuprates at -78°C gave vinyl sulfone 3 in excellent yields with cis:trans

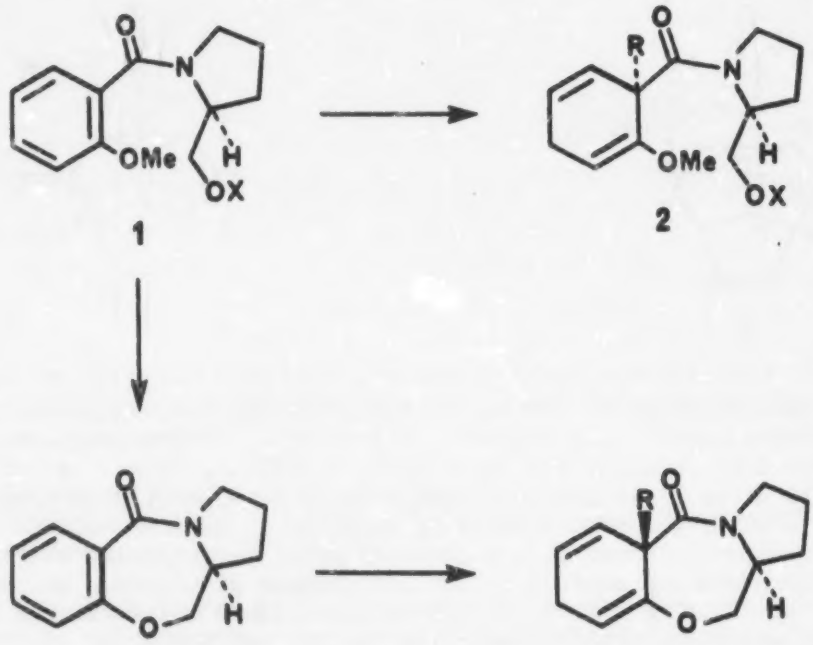


ratios better than 95:5. Alternatively, direct treatment of amine 1 with organolithium reagents afforded 4, which upon quaternization and elimination afforded 5 in good overall yields with trans:cis ratios again better than 95:5.

This appears to be a general approach, as it has been applied successfully to the synthesis of 3 and 5 with R = methyl, phenyl, allyl, vinyl, t-Butyl, and alkynyl.

"Total Synthesis of Natural Products"
RO1 GM 26568-07 (Schultz, A.), Rensselaer Polytechnic Institute

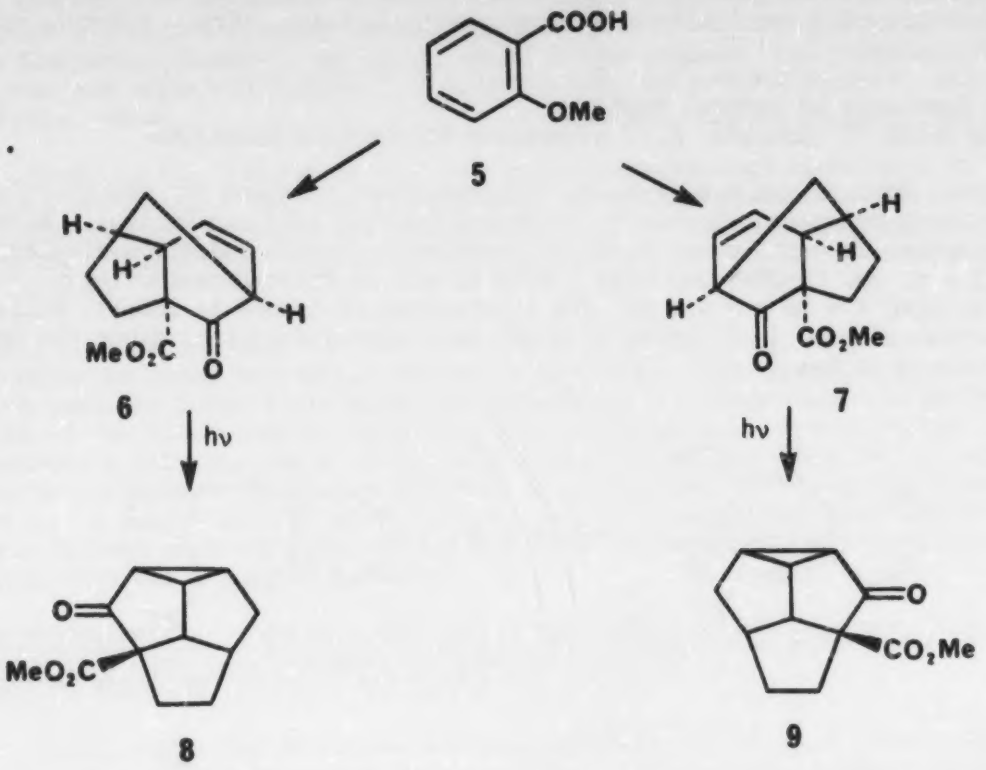
Dr. Arthur Schultz and his students have developed a method for enantioselection in the Birch reduction-alkylation of chiral benzoic acid derivatives. Benzamide 1 is prepared from salicylic acid and L-prolinol. Birch reduction-alkylation of 1 (X = H, Me, CH_2OMe) provides 2 with excellent diastereoselectivity (for example, with R = Me and X = Me, the diastereomeric excess is 260:1), while cyclization of 1 to 3, followed by Birch reduction-alkylation, gives the opposite enantiomeric series 4.



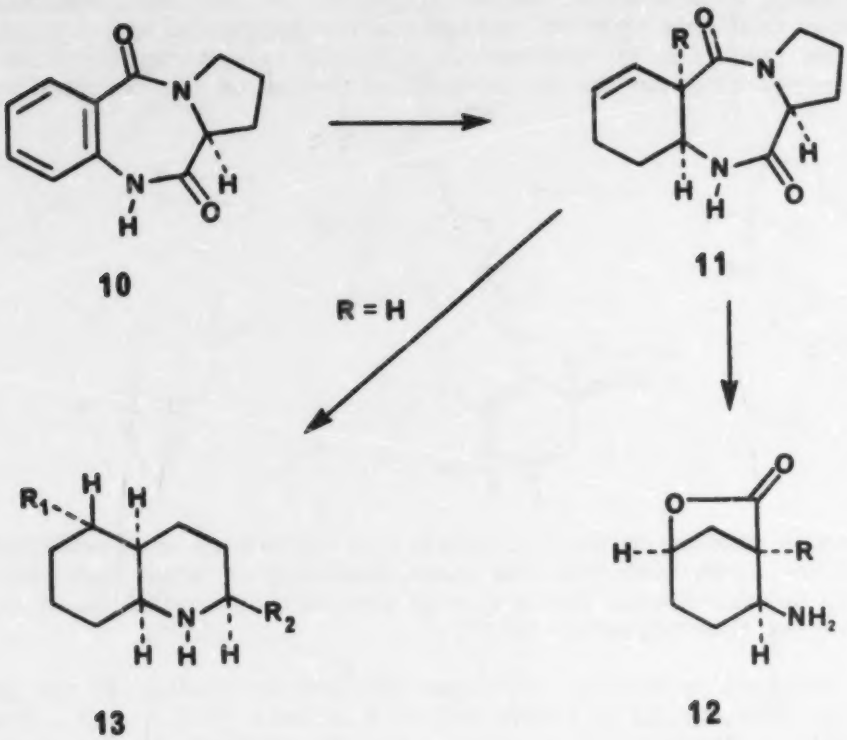
1 2

3 4

This methodology, together with developments in 2,4-cyclohexadien-1-one chemistry, have resulted in the efficient synthesis of a wide range of structural types for use in the total synthesis of medicinally important natural products. For example, 5 is converted to enantiomerically pure intramolecular Diels-Alder adducts 6 and 7, and these undergo oxa-di-π-methane photorearrangement to 8 and 9, respectively. A modification of this chemistry leads to construction of the cytotoxic (+)-and (-)pentalenolactones.



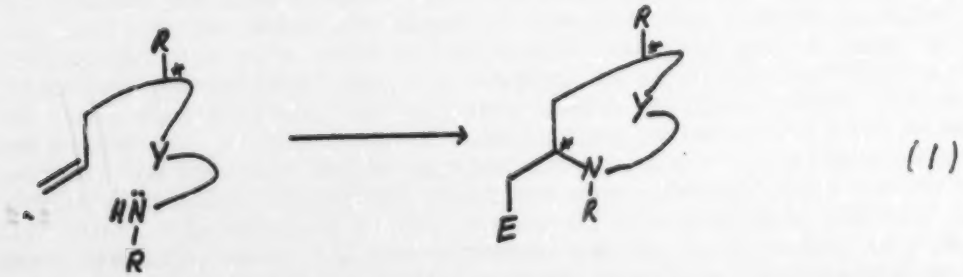
The enantioselective Birch reduction-alkylation has been extended to other aromatic and heteroaromatic ring systems. For example, anthranilic acid derivative 10 is prepared by reacting isatoic anhydride with L-proline. Birch reduction-alkylation of 10 gives 11 in good to excellent yields without producing diastereoisomers. The chiral auxiliary can be removed by treating 11 with 50 percent aqueous sulfuric acid. Pure amino lactones 12 are obtained after partitioning reaction components between chloroform and aqueous sodium bicarbonate solutions. Dr. Schultz has used this technique (R = H) in the enantiospecific construction of several pumiliotoxins, 13, which are lipid-soluble ion channel antagonists.



The conversion of readily available aromatic substrates to chiral synthetic intermediates, both cyclic and acyclic, should provide access to a diverse array of natural product targets.

"Stereoselective Synthesis of Polyfunctional Amines"
R01 GM 31834-02 (Harding, K.), Texas Agricultural and Mechanical University, College Station

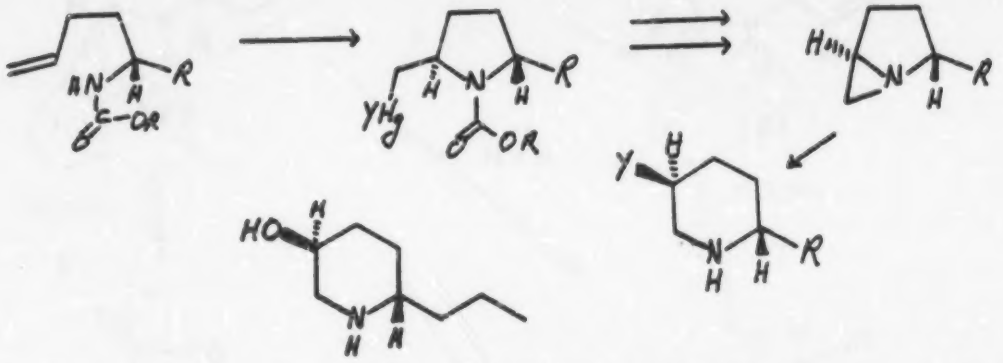
Dr. Kenneth Harding is developing new methods for the stereoselective synthesis of both cyclic and non-cyclic amines of biological importance. He has investigated ring-forming reactions resulting from electrophile-initiated addition of a nitrogen nucleophile to a carbon-carbon double bond (reaction 1).



(1)

Using mercuric salts as the electrophile and carbamates as the nitrogen nucleophile, he has accomplished the stereoselective synthesis of trans-2,5-dialkylpiperidines

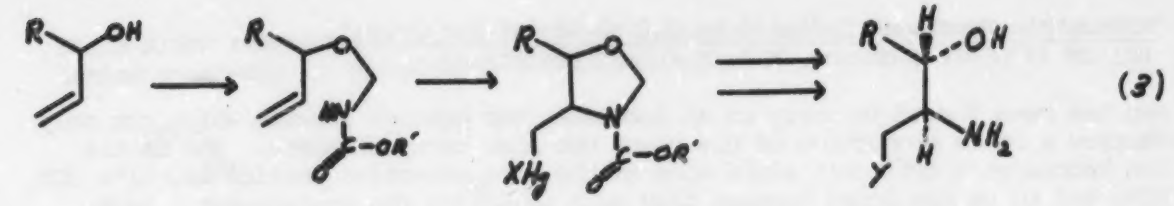
with extremely high stereoselectivity (98:2). He has used organomercurial intermediates from this type of cyclization for synthesizing trans,-2,5-disubstituted piperidines (reaction 2); the hemlock alkaloid (±)-pseudoconhydrine was synthesized in this manner to prove the stereochemical course of the reaction sequence.



(2)

Dr. Harding's studies on the cyclization of carbamates with mercuric salts have shown for the first time that the stereochemistry of these cyclizations can be critically dependent upon the choice of cyclization conditions, indicating kinetic control versus thermodynamic control.

The most interesting results have been obtained in studies of the use of the cyclization strategy for stereoselective synthesis of 1,2- and 1,3-aminoalcohols from allylic or homoallylic alcohols. Allylic alcohols can be stereoselectively converted to threo 1,2-aminoalcohols (reaction 3), and homoallylic alcohols can be converted into either threo or erythro 1,3-aminoalcohols (reaction 4). These reactions will allow development of new methods for stereoselective synthesis of alkaloids, uncommon amino acids, and other polyfunctional amines.



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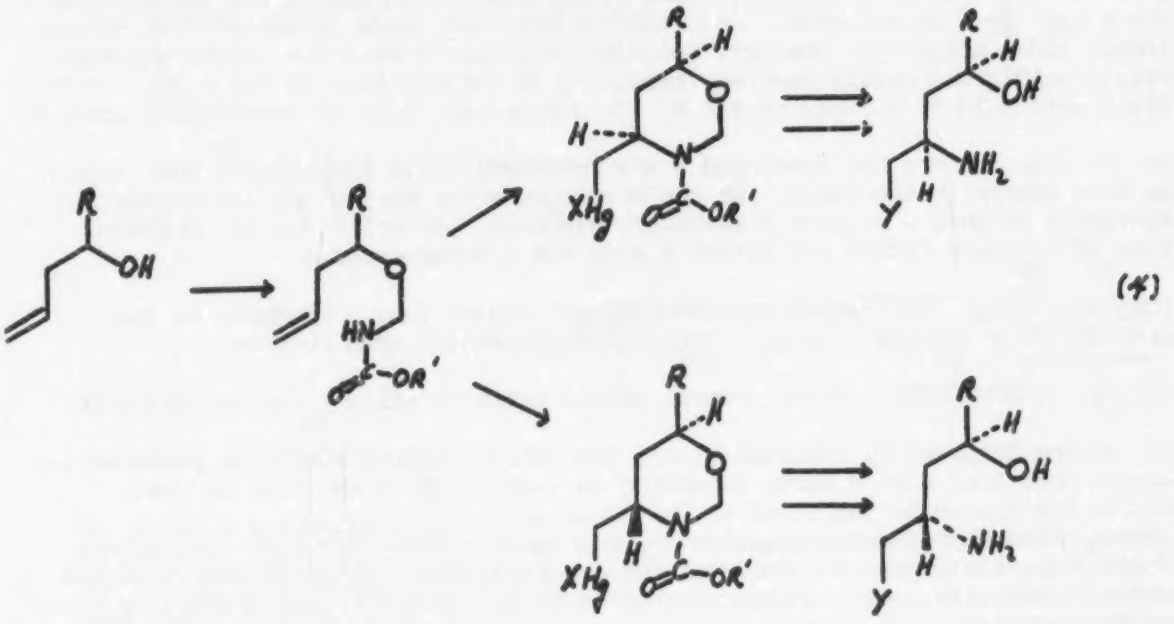


(4)

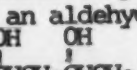
"Stereospecific Synthesis of β-Amino Alcohols"
R01 GM 33230-01A1 (Gassman, P.), University of Minnesota

Dr. Paul Gassman's investigations have been aimed at assessing the scope of a recently discovered reaction that involves the trans opening of epoxides with trimethylsilyl cyanide and zinc iodide. This reaction yields trimethylsilyl ethers of β-hydroxy isonitriles, which are readily converted into β-amino alcohols. Careful examination of a wide range of Lewis acid catalysts over a range of concentrations has demonstrated that the reaction is extremely dependent on the metal used: only zinc (II) and tin (II) were useful catalysts, with zinc derivatives being preferred. A wide range of substitution patterns could be tolerated for the epoxide; the reaction can be carried out on relatively unhindered epoxides with dimethyl-t-butylsilyl cyanide to yield the more hindered protected dimethyl-t-butylsilyl ether. In addition to the opening of epoxides, this reaction similarly cleaves oxetanes, which are four-membered analogs of epoxides, to yield silylated γ-hydroxy isonitriles. Utilizing these reactions, amino alcohols can be produced that would be extremely difficult to prepare by other existing processes.

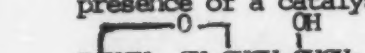
"Asymmetric Syntheses Mediated by Chiral Acetal Templates"
R01 GM 33766-01 (Johnson, W.), Stanford University

Man has been forced to carry on an incessant war against insects which not only destroy a large proportion of his crops but also carry diseases. The battle has become more difficult since some of the more effective pesticides, like DDT, have had to be abandoned because they were polluting the environment. Among the biodegradable insecticides, the pyrethroids, e.g., Deltamethrin, are now some of the most important. These are esters of α-cyano-3-phenoxybenyl alcohol, an asymmetric (chiral) molecule that exists in two mirror image forms, the R and the S enantiomers. Only the S enantiomer leads to esters with strong insecticidal activity. However, the α-cyano group renders the α-hydrogen atom fairly acidic and easily removed, resulting in racemization of the S form in the field into a 50-50 mixture of S + R with concomitant loss of insecticide activity.

Dr. William Johnson has developed a new pyrethroid-like insecticide that should be more stable in the field. He has developed a new method for the asymmetric synthesis of pure R or pure S secondary alcohols. A cyclic acetal, produced from an aldehyde (RCHO) and either R,R or S,S 2,4-pentanediol



($CH_3CHCH_2CHCH_3$), is treated with a Grignard reagent (e.g., CH_3MgCl) in the presence of a catalyst ($TiCl_4$). The Grignard reagent adds to give



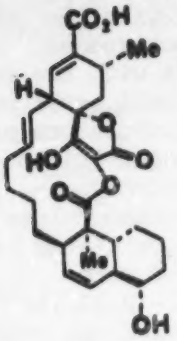
$RCHCH_3$ $CH_3CHCH_2CHCH_3$. After removal of the chiral auxiliary (by oxidation to the ketone followed by β-elimination), the chiral alcohol $RCHCH_3$ is produced in almost pure R or pure S form, depending on which form of the diol is used.

When applied to 3-phenoxybenzaldehyde, both enantiomeric forms of α-methyl-3-phenoxybenzyl alcohol were produced for the first time. These differ from the alcohol generally used to make pyrethroids in that there is an α-methyl in place of the α-cyano group. The α-methyl group in the new alcohols does not render the α-hydrogen acidic, so these substances do not racemize readily. The 3-methyl-2-(4-chloroanilino)-butyrate esters of the two new alcohols were prepared and tested for insecticidal activity. One of these esters was almost devoid of insecticidal activity, while the other was a potent insecticide. Comparison of LD_{50} values with those for the analogous known commercial esters derived from the α-cyano alcohols indicated that the new active α-methyl pyrethroid was more effective against the common house fly (Musca domestica (LD_{50} = 0.13 μg/insect) than against the tobacco budworm Heliothis virescens (LD_{50} = 0.085). The next step will be to find out if this α-methyl pyrethroid has the expected stability in the field.

"Intramolecular Diels-Alder Reactions"
R01 GM 26782-06 (Roush, W.), Massachusetts Institute of Technology

Dr. William Roush and his colleagues have explored two new Diels-Alder methodologies directed toward the total synthesis of the antibiotic chlorothricolide, 1. First, they developed an enantioselective synthesis of the bottom-half component 2, featuring the intramolecular Diels-Alder reaction of 3. The bromine substituent, a "steric directing group," functions here as a strategic vehicle for stereochemical control. Second, they synthesized the top-half synthon 4 by the novel

Diels-Alder reaction of triene 5, which possesses an enol pyruvate unit. The latter synthesis is potentially enantioselective.



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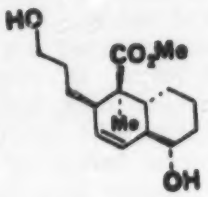

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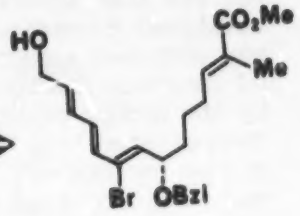

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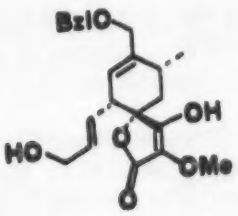

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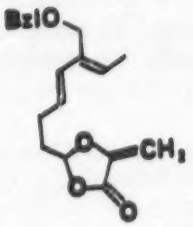

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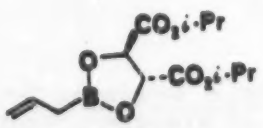
Dr. Roush and coworkers also have explored the reactions of crotyl and allylboronic esters with chiral aldehydes in an attempt to develop a set of chiral reagents for controlling aldehyde diastereofacial selectivity. Very exciting results have been realized with reagents that incorporate tartrate esters as the chiral auxiliary, as illustrated below for reactions involving glyceraldehyde acetonide.



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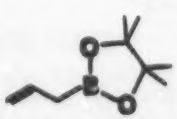

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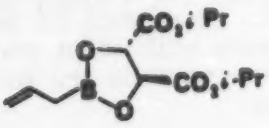

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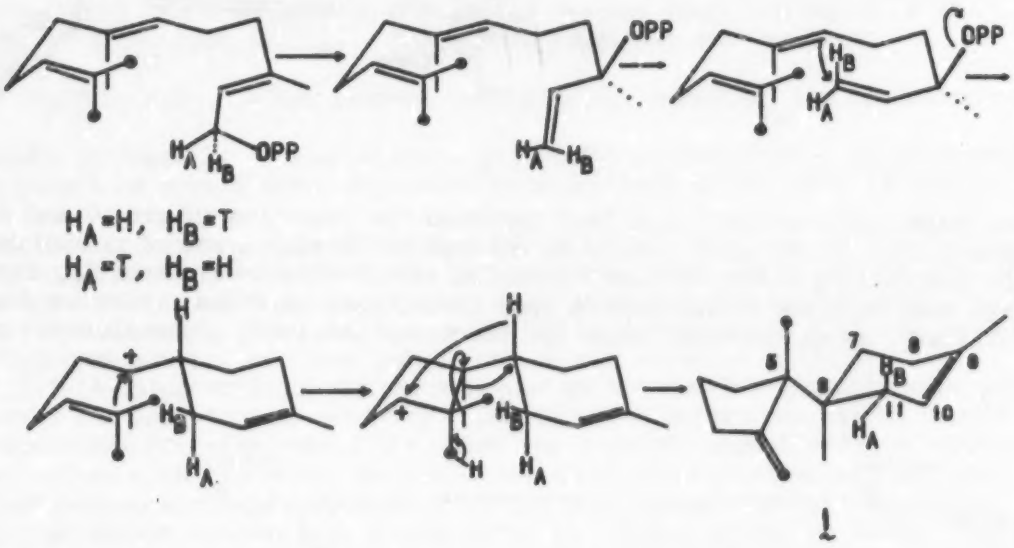
"Stereochemical Studies of Isoprenoid Biosynthesis"
R01 GM 30301-04 (Cane, D.), Brown University

For the last several years, Dr. David Cane's group has been studying the enzyme-catalyzed formation of a group of cyclic sesquiterpenes and monoterpenes. He has proposed a mechanism, illustrated below, which is consistent with the results of numerous incorporation experiments carried out at the whole cell level primarily by the groups of Hanson, Nozoe, and Tamm. Dr. Cane and his collaborators previously demonstrated that trichodiene synthetase from the apple mode fungus Trichothecium roseum can convert [1-$^{3}H_2$,12,13-^{14}C]farnesyl pyrophosphate to trichodiene, 1, the parent hydrocarbon of the trichothecane family of antibiotics, without loss of tritium label. They have obtained further data to support the postulated isomerization-cyclization mechanism by demonstrating that cyclization of both 1R-[1-^{3}H,12,13-^{14}C]farnesyl pyrophosphate (H_A=H, H_B=T) and 1S-[1-^{3}H,12,13-^{14}C] farnesyl pyrophosphate (H_A=T, H_B=H) to trichodiene takes place with net retention of configuration at C-1 of the farnesyl pyrophosphate precursor. The distribution and stereochemistry of labeling was confirmed in each case by chemical degradation of the derived labeled trichodiene.

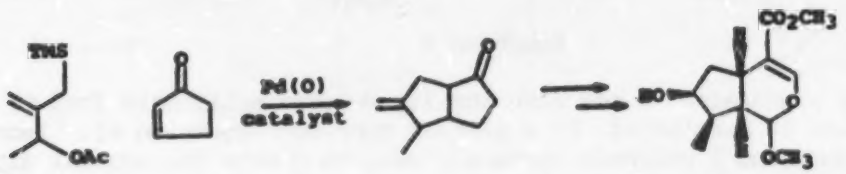


Cyclic Synthesis

"Prostaglandins, Steroids, Macrolides, etc.: Synthetic Designs"
R01 GM 13598-19 (Trost, B.), University of Wisconsin, Madison
"Synthesis of Biologically Active Cyclopentenones"
R01 GM 26294-06 (Schore, N.), University of California, Davis

Many therapeutically important or promising compounds possess five-membered rings as a key portion of their structure. Thus, the design and synthesis of substituted five-membered ring compounds is important. Synthesis of the cyclopentane ring system poses some special problems in common with other rings of odd size, so that most syntheses of five-membered ring compounds are longer and less efficient than syntheses of even-sized ring systems.

Dr. Barry Trost has created a process for making rings other than those containing six members, using a novel silicon reagent and a special catalytic template. Equation 1 illustrates the use of this method in providing an easy entry to loganin.



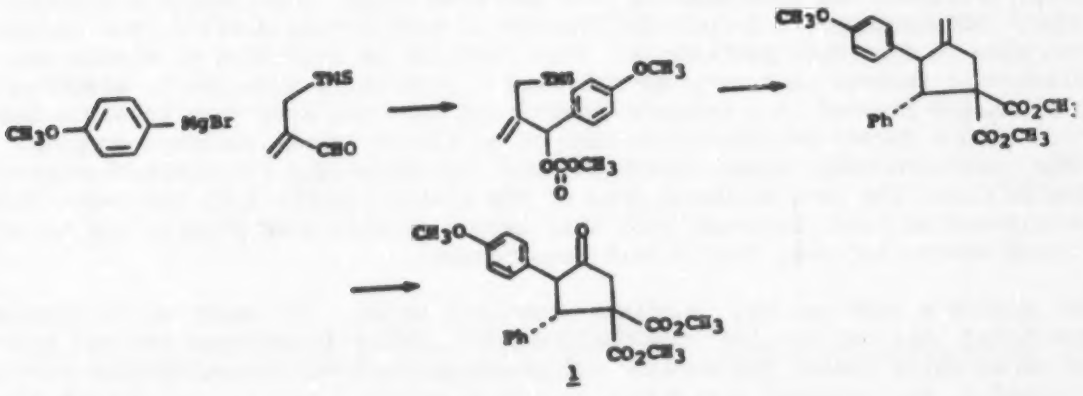
(1)

loganin aglucone CH_3 ether

Equation 1

The key to Dr. Trost's approach is the stabilization of a short-lived intermediate via its complexing to a metal template. This efficient cycloaddition to five-membered rings has the same range of selectivity as the Diels-Alder reaction.

The facility of this method is illustrated by an approach to rocaglamide, in which the cyclopentanone 1 is a critical intermediate. The requisite substrate for cycloaddition is the newly created conjunctive reagent 2-trimethylsilylmethyl acrolein, shown in Equation 2. In this reaction, the exocyclic methylene group serves as a structural equivalent of the carbonyl group of 1.

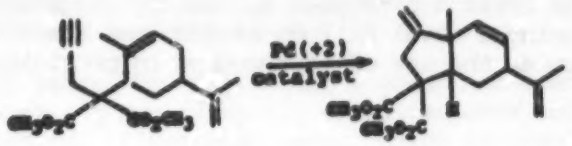


1

(2)

Equation 2

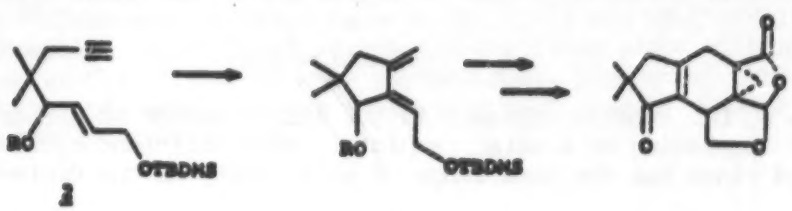
A totally different catalytic reaction offers a new strategy for the creation of five-membered rings by isomerizing a linear chain into a ring as in Equation 3. This route easily creates the ring system of the picrotoxins from carvone, an inexpensive and readily available starting material.



(3)

Equation 3

Dr. Trost has synthesized a new structurally related antibiotic family, one member of which is merulidial, by a similar approach (Equation 4). Isomerizing the acyclic compound 2 proceeds extremely well to create the unusual dimethylene-cyclopentane, from which the final target, sterepolide, is produced in only eight



(4)

Equation 4

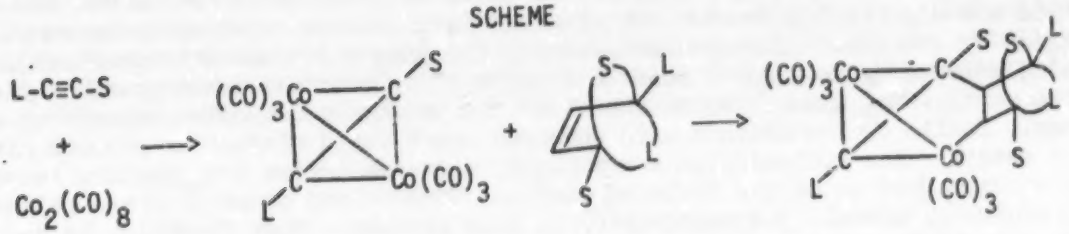
steps. These two methods should form a powerful basis for synthesizing biologically important cyclopentanoid natural products and analogs.

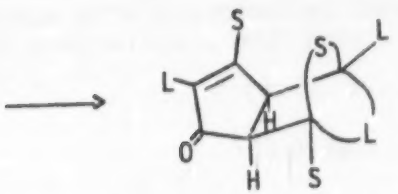
Dr. Neil Schore and his colleagues have been exploring the usefulness of transition metal catalysis for synthesizing five-membered rings, including the ability of cobalt octacarbonyl to induce cyclization of acetylenes, olefins, and carbon monoxide to form cyclopentenones. This reaction is sensitive to steric and electronic factors in a very specific way. Sterically unhindered, electron rich olefins are favored in a product-determining reaction step that involves insertion into a formal cobalt-carbon bond of an alkyne-cobalt carbonyl complex. In this insertion step, which determines the regiochemistry and stereochemistry of the product, the less hindered face of the olefin inserts into the least hindered cobalt-carbon bond, oriented such that larger allylic substituents are directed toward carbon and away from cobalt (see Scheme).

Dr. Schore's work has had immediate practical value. For example, he accurately predicted that two cyclizations designed to generate precursors for the synthesis of medicinally useful guaianolide and pseudoguaianolide natural products would proceed in the required directions to afford enones 1 and 2. Dr. Schore also predicted that compound 3, a possible precursor for triquinanes such as the anti-cancer agent coriolin, would not be easily accessible; therefore, he chose an alternative route for this purpose.

Knowledge gained in Dr. Schore's research has led to the design of extremely efficient intramolecular synthetic routes to a variety of polyquinane systems with a high degree of control over critical centers of stereochemistry. A notable recent example is the successful preparation and cyclization of enyne 4 to form triquinane 5, with control of the stereochemistry of the (starred) 2° methyl substituent. Reactions such as this have great potential in the synthesis of a number of highly active compounds, including cytotoxic agents of the pentalenolactone/pentalenic acid series.

SCHEME

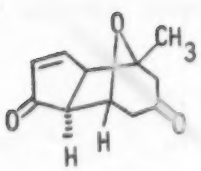




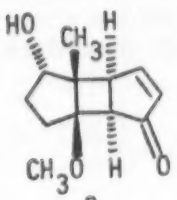
preferred product

1. exo ring fusion
2. L on alkyne α to new C=O
3. L allylic to olefin anti to new C=O

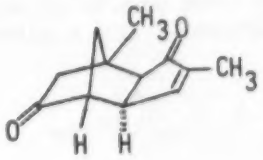
(L = large group; S = small group)



1



2



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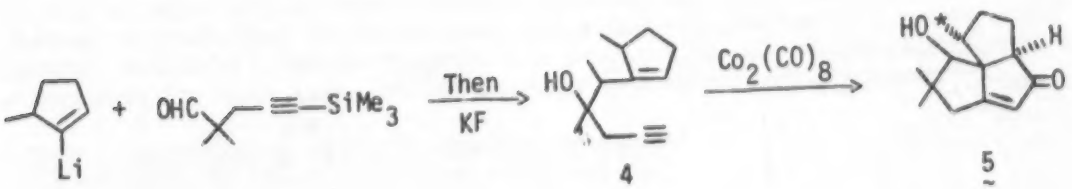


4

5

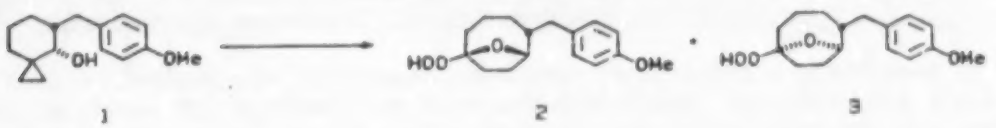
"Intramolecular Nitrosoalkene-Olefin Cycloadditions"
R01 GM 30938-03 (Denmark, S.), University of Illinois, Urbana-Champaign

Dr. Scott Denmark is evaluating the scope and generality of cycloadditions based on the N=O group in creating polycyclic ring systems with regio- and stereocontrol. He also is evaluating the compatibility of the approach with various functional groups, the potential for synthetic manipulation of the products, and the ease of preparation of the precursors. He finds that nitrosoalkenes are generated from easily prepared α-chloro oximes and undergo smooth cyclization to form dihydrooxazines, with concomitant construction of five-, six-, and seven-membered rings. He has discovered that enol ethers, especially thio enol ethers, are excellent dienophiles for this reaction.

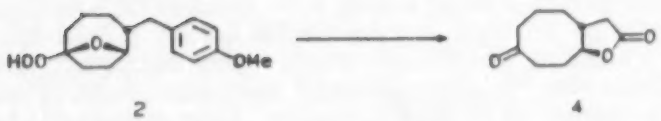
Vinyl nitrosonium cations generated in situ from α,β-epoxy nitrones undergo cycloaddition with appended, unactivated olefins. The reactions succeed in both cyclic and acyclic frameworks. Dr. Denmark has obtained crystalline tetrahydrooxazine by treatment with cyanide, thereby creating three new stereocenters in a single isomer with greater than 10:1 selectivity over all other possibilities. Subsequently, Dr. Denmark found that both cis and trans nitroalkenes undergo extremely facile cycloadditions with appended unactivated olefins to produce nitronate esters. The cycloadditions are highly stereoselective for creating isomers via an exo- folding of the tethered olefin. Double-bond geometries are reflected faithfully in overall stereochemistry in this process. Most recently, he has been able to generate a tandem cycloaddition sequence by employing the nitronate product of the first process as a dipole in a second cycloaddition with acrylate esters. The product is a novel tricyclic compound with five stereocenters, each established by a pericyclic process.

"Solvolytic Hydroperoxide Rearrangements"
R01 GM 33034-02 (Ronald, R.), Washington State University

Dr. Robert Ronald has discovered that hydrogen peroxide-mediated ring expansion of carbinol 1 is a stereoselective process that produces the cyclooctanone hemiketals 2 and 3 in good yield with 8:1 selectivity. Bicyclic hydroperoxides 2 and 3 were easily separable and could be converted to the keto-lactones 4 and 5, respectively, by a simple and direct procedure.



1 2 3



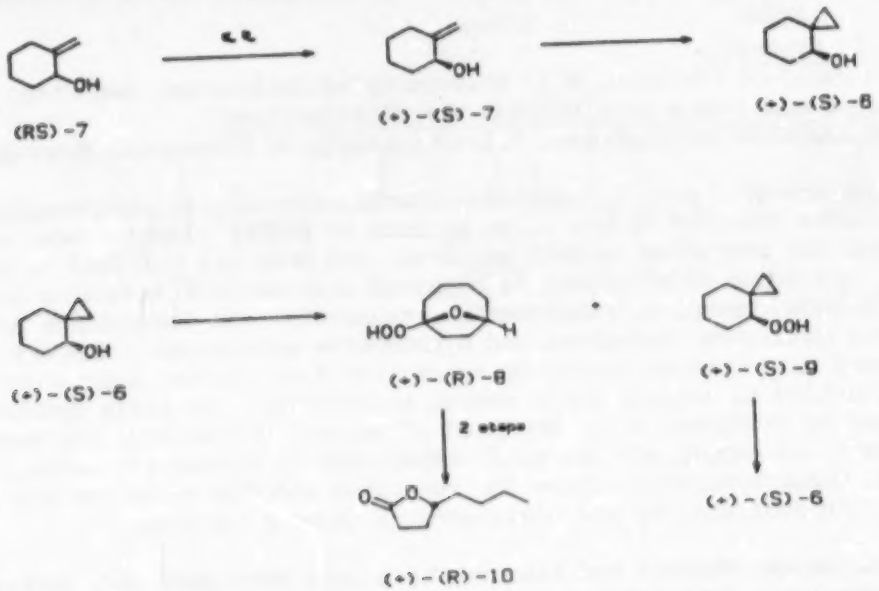
2 4

3 5

X-ray structures of the crystalline keto-lactone from the major ring expansion product and of the major ring expansion product itself unequivocally show the structures of the major isomers to be 4 and 2, respectively. Fortuitously, this ring expansion selectively produces the trans fused lactones, which are the most commonly encountered forms among natural products of synthetic, biological, and pharmaceutical interest but are the most difficult to obtain.

The stereoselective formation of 2 requires frontside migration of the syn methylene of the cyclopropane ring during the ring expansion process, a mode of migration that would not have been expected to be so stereoselective based upon other investigations of cyclopropylcarbinyl systems.

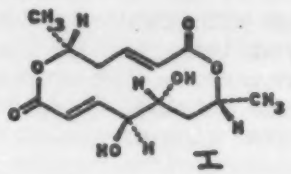
To establish the primacy of ion stereochemistry, Dr. Ronald prepared carbinol 6 in optically pure form with known absolute configuration. Using the Kinetic Resolution method of Sharpless, (+)-(S)-2-methylene cyclohexanol 7 was readily obtained from racemic material. Cyclopropanation using CH_2I_2-Zn(Cu) afforded (+)-(S)-6, which could be obtained in optically pure state by recrystallization. Hydrogen peroxide-mediated ring expansion under conditions that preserved the cyclopropylcarbinyl hydroperoxides produced dextrorotatory 8 and 9:



These results strongly support a common asymmetric ionic intermediate as a central feature of these rearrangements and demonstrate that frontside migration is the normal mode of these hydrogen peroxide mediated rearrangements. They further suggest that these hydrogen peroxide-mediated rearrangements could be a general method for the stereoselective preparation of complex multifunctional structures for synthetic, biological, and pharmaceutical purposes.

"Chiral Approaches to Natural Product Synthesis"
R01 GM 28961-04 (Keck, G.), University of Utah

Dr. Gary Keck has improved the procedures for constructing large ring, or macrocyclic, lactones such as those found in erythromycin and other important antibiotics. A very useful procedure for bimolecular esterification, previously described by Steglich, was examined in this context but, despite a variety of attempts, produced very low (4-5 percent) yields of macrocyclic lactones from long chain ω-hydroxy acids. Dr. Keck critically examined the mechanistic aspects of this procedure and determined the structure of the major by-product produced. He found that the low yield probably was due to decreased rates of certain key proton transfer steps under the high dilution conditions appropriate for macrolactonization. By simply including certain amine hydrochlorides in the reaction to assist the proton transfer steps, yields of macrolactone were as high as 95 percent. Dr. Keck has found this new process to be useful in the synthesis of the antibiotic colletodiol (see structure below), and could be generally useful in the synthesis of complex macrolide antibiotics.



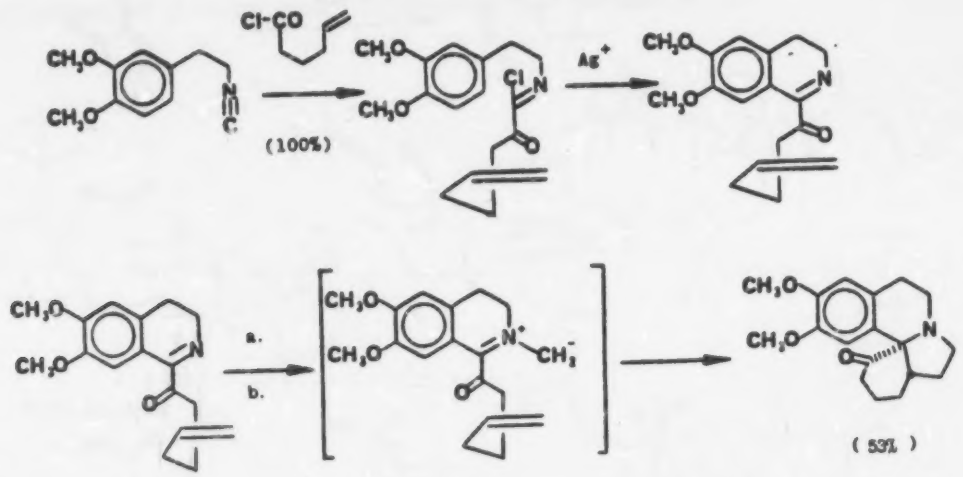
I

"Alkaloid Synthesis"
R01 GM 29232-05 (Wenkert, E.), University of California, San Diego
"Synthetic Approaches to Alkaloids Via Nitrile Ylids"
R01 GM 32000-03 (Livinghouse, T.), University of Minnesota, Minneapolis

Alkaloids are structurally complex, nitrogen-containing, pharmacologically active constituents obtained from a large variety of higher plants. Some alkaloids have been used for centuries in folk medicine, and some are utilized as present-day drugs. One group of alkaloids is obtained from various Rauwolfia species; these include reserpine, rescinnamine, raunescine and deserpidine and are known to be tranquilizers, sedatives and hypotensive substances. Whereas these drugs at present are obtained mainly by extraction from plants, they occur in insufficient quantity to satisfy their demand in medicine. An early synthetic process, developed by Professor R. B. Woodward of Harvard University, has been used to supplement the supply of reserpine, especially in France and eastern Europe. However, other synthetic routes for reserpine and its relatives are needed to broaden the availability and usefulness of these materials.

Professor Ernest Wenkert and his coworkers have developed new, shorter synthetic procedures for a large number of bioactive alkaloids. Last year, they completed development of a 13-step synthesis of deserpidine and a 14-step construction of raunescine, both of which have hypotensive properties. Readily available nicotinaldehyde served as the starting materials. These syntheses open up new routes for modifying structures and hence may lead to the development of new pharmacologically active agents.

During the last year, Dr. Thomas Livinghouse has successfully perfected two highly general methods for synthesizing heterocycles. One method generates highly reactive cationic species called nitrilium ions. These intermediates undergo cyclization reactions with internal carbon-based nucleophiles to produce heterocycles in high yield. The other synthetic method involves a new way to construct functionalized pyrrolidine rings, using intermediates known as stabilized iminium ylids. Both of these new methods have been used in tandem to synthesize the skeletal system of the Erythrina alkaloids in just two steps (Scheme I).

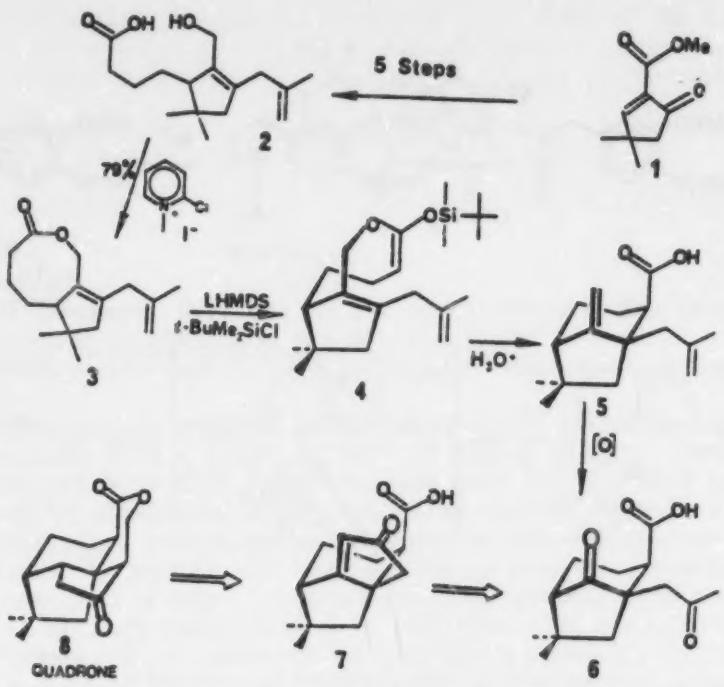


a.) $Me_3SiCl_2O_3SCF_3$ b.) CsF

SCHEME I

"New Cyclization Methods for Natural Product Synthesis"
R01 GM 28663-05 (Funk, R.), University of Nebraska, Lincoln

Dr. Raymond Funk and his students have been developing new strategies for hetero- and carbocycle synthesis. They recently completed a new approach for the total synthesis of quadrone, 8, a structurally complex natural product that has antitumor properties.

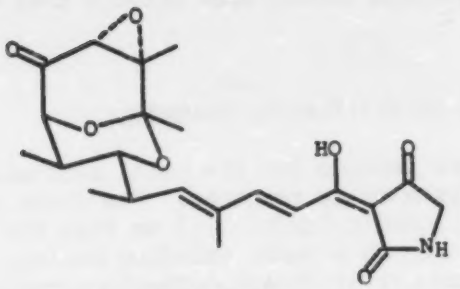


The acid 5 was efficiently prepared by an application of Danishefsky's and Schlessinger's method for carbocycle construction. The hydroxy acid 2 was easily obtained in five steps from the cyclopentenone 1 and was lactonized to provide the eight-membered lactone 3. Rearrangement of lactone 3, via intermediate ketene acetal 4, proceeded at or below room temperature to deliver, stereospecifically, the substituted bridged bicyclooctane 5. Conditions were discovered for oxidizing 5 to the diketo acid 6 in order to complete the formal total synthesis of quadrone. They now are investigating variations of this new approach to bridged bicycloalkanes, which should provide novel and convenient routes to a variety of biologically active compounds.

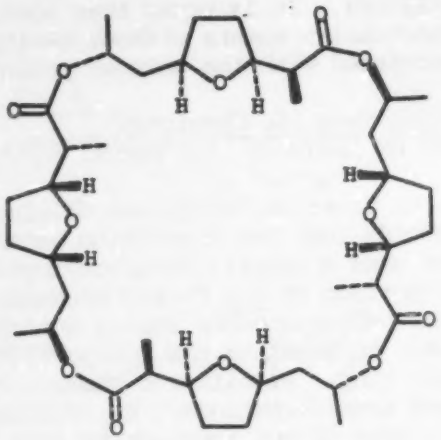
"Synthesis of Macrocyclic Natural Products"
R01 GM 30759-11 (Bartlett, P.), University of California, Berkeley

Dr. Paul Bartlett is exploring model reactions which facilitate the introduction of specific stereocenters into acyclic precursors. Specific methods that he has developed include iodolactonization for the controlled epoxidation of olefinic carboxylic acids, and iodocyclization of unsaturated phosphates, carbonates, and carbamates for functionalization of olefinic alcohols. These reactions make available in stereochemically pure form the building blocks for the total synthesis of compounds such as the anti-tumor antibiotic tirandamycin and the macrolide antibiotic nonactin (structures displayed below).

This approach to the chemical synthesis of such compounds not only extends knowledge of synthesis, but it also makes available labeled and modified derivatives of some biologically significant compounds for further study.



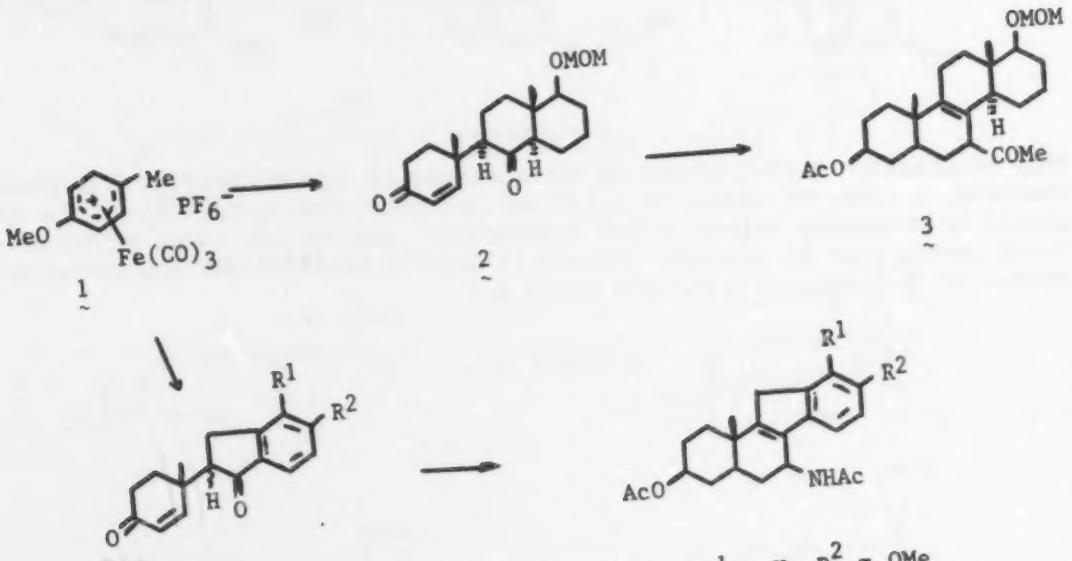
Tirandamycin



Nonactin

"Steroid, C-nor-D-homosteroid and Aphidicolin Synthesis"
R01 GM 30373-03 (Pearson, A.), Case Western Reserve University

Dr. Anthony Pearson has developed a convergent approach for steroid synthesis. By this process, iron complex 1 is converted to enone 2 in four steps and 2 is then converted to the D-homosteroid 3.



4 R^1 = H, R^2 = OMe
R^1 = Me, R^2 = H

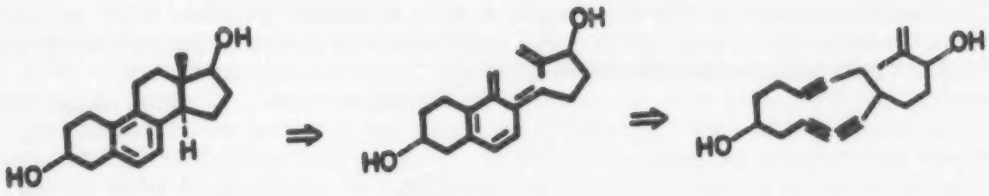
5 R^1 = H, R^2 = OMe
R^1 = Me, R^2 = H

He is developing a means to synthesize 7-substituted Veratrum alkaloids, which are potential antihypertensive agents. Toward that goal, Dr. Pearson has converted complex 1 through enones 4 to C-nor-D-homosteroids 5, which should be useful intermediates for the synthesis of unusual 7-amino substituted Veratrum alkaloids. It is hoped that some of the synthetic products might serve as antihypertensive agents without having the unfortunate emetic side effects that are associated with the natural products.

"Organometallic Chemistry"
R01 GM 22479-10 (Vollhardt, K.), University of California, Berkeley

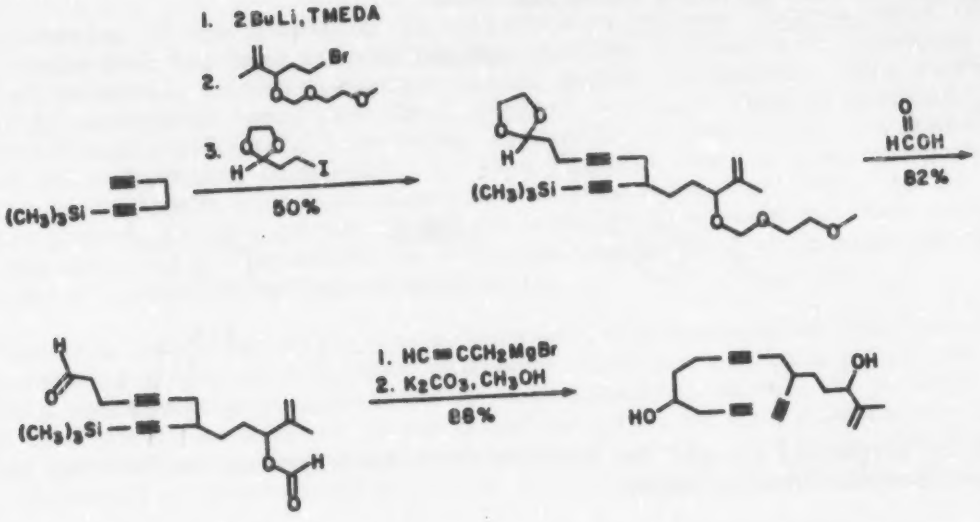
Dr. K. Peter Vollhardt has developed three new methods for the total synthesis of steroids that use transition metals as catalysts or as reagents. The first procedure uses catalytic dicarbonylcyclopentadienylcobalt [$CpCo(CO)_2$] to fuse the ABC-portion of the A-ring aromatic steroids onto an already existing D-ring. The other two approaches employ stoichiometric amounts of dicarbonylcyclopentadienylcobalt to annelate the BCD-portion of the steroids to an already existing A-aromatic ring. Significant chemo-, regio-, and stereoselectivity is achieved in these transformations. Dr. Vollhardt and his coworkers have now found an even more direct approach to this physiologically important class of molecules, in which an enetriyne is cyclized with catalytic cobalt in one step to assemble all four rings. The retrosynthetic analysis is shown in Scheme I.

Scheme I



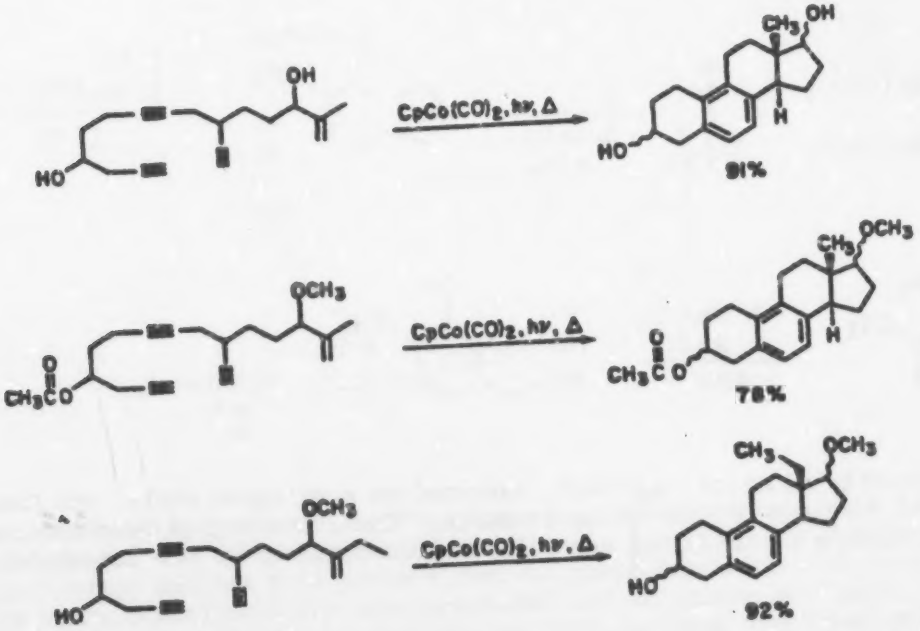
This cyclization gives rise to an extraordinarily rapid assembly of B-aromatic steroids, a class of compounds which was not previously accessible. The approach should be extremely versatile for synthesizing analogs and novel medicinal agents based on the steroid nucleus. Scheme II depicts an efficient and convergent synthesis of the required enetriyne percursor.

Scheme II



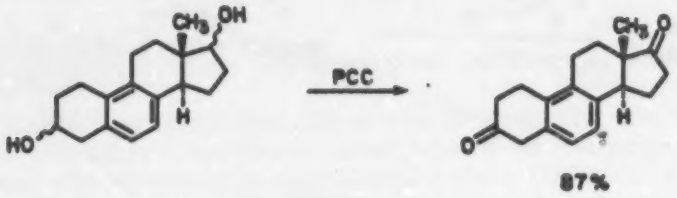
The examples in Scheme III demonstrate the efficiency and versatility of the method. Target molecules are assembled with complete stereoselectivity with respect to the CD-ring junction, which emerges trans, as in the naturally occurring systems.

Scheme III



Dr. Vollhardt used spectral and chemical techniques to identify the cyclization products. As shown in Scheme IV, oxidation of the B-ring aromatic 3,17-diol gives the corresponding known diketone:

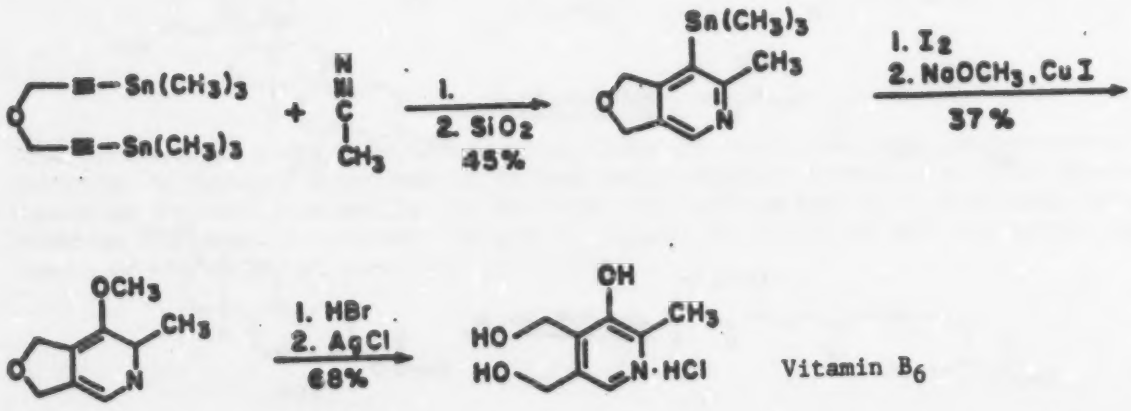
Scheme IV



He is preparing several derivatives of these compounds for testing in an androgen receptor binding assay.

By utilizing his previous finding that cyclizations catalyzed by dicarbonylcyclopentadienylcobalt are not restricted to unsaturated moieties containing only carbon, Dr. Vollhardt has developed a highly chemo- and regioselective construction of vitamin B_6 (Scheme V).

Scheme V



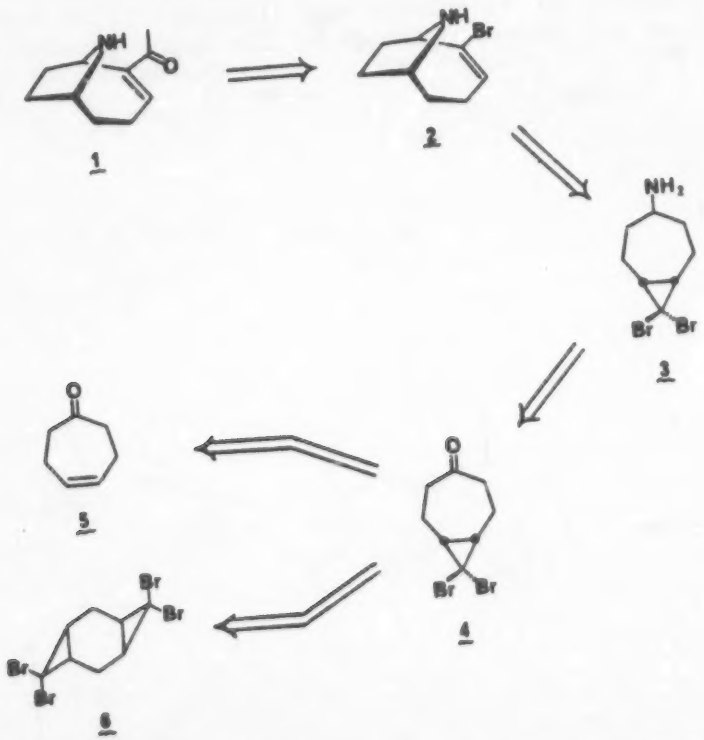
Vitamin B_6

A conceptually identical approach, executed on a kilogram scale, was used recently by the Hoffmann-LaRoche group in Basle. Thus, strategies developed in Dr. Vollhardt's laboratories are suitable for scale-up to the industrial level.

"Synthetic Approaches to Cyclic Natural Products"
R01 GM 25950-07 (Danheiser, R.), Massachusetts Institute of Technology

Certain strains of the freshwater blue-green alga Anabaena flos-aquae produce a potent toxin that has been responsible for numerous incidents of livestock and waterfowl poisoning in the midwestern United States and Canada. This substance, originally designated "very fast death factor" but later renamed anatoxin a (2-acetyl-9-azabicyclo[4.2.1]non-2-ene, 1) rapidly causes death in a number of species via respiratory paralysis. It is a powerful depolarizing neuromuscular blocking agent, possessing both muscarinic and nicotinic activity. As one of the most potent agonists of the nicotinic acetylcholine receptor discovered to date, the compound has proved to be a valuable research tool in elucidating the mechanism of intramuscular neurotransmission.

Professor Rick Danheiser and his coworkers at the Massachusetts Institute of Technology have developed an efficient synthetic route for anatoxin a that can be used to prepare multigram quantities of this alkaloid, as well as certain derivatives with potential utility in the chemotherapy of nervous disorders. The pivotal step in his approach, outlined in the following scheme, is the disrotatory electrocyclic cleavage-transannular cyclization of the bicyclic amine 3,

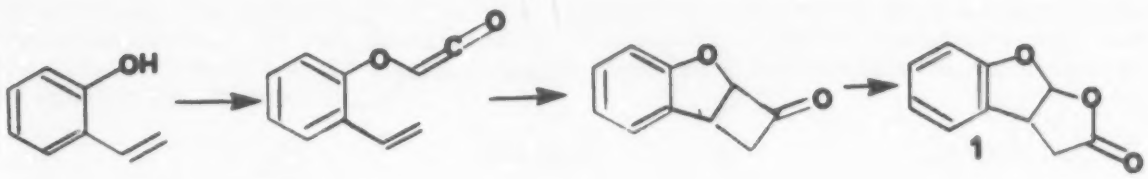


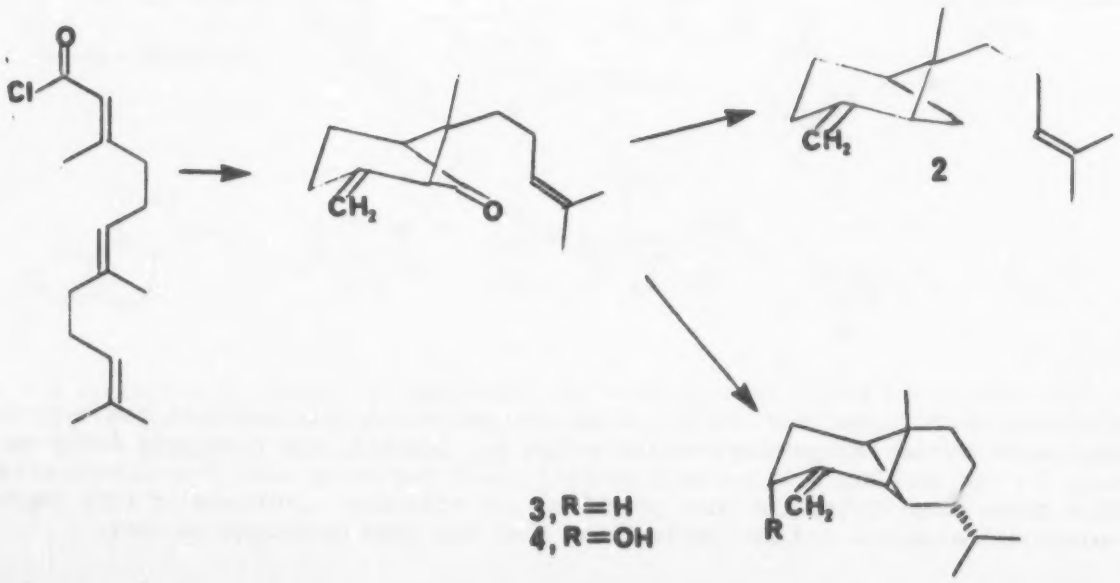
which is readily produced via two alternate routes starting with either 4-cycloheptenone or the tricyclooctane derivative 6. Overall, the synthesis involves only 7 steps and has a 17 percent overall yield beginning with 4-cycloheptenone. This route constitutes the most practical and efficient synthesis of this important neurotoxic alkaloid and its derivatives that has been developed to date.

"New Carbon-Carbon Bond Forming Reactions of Alkenes"
R01 GM 30528-05 (Snider, B.), Brandeis University

Dr. Barry Snider and coworkers have developed a variety of Lewis acid catalyzed and induced reactions of carbon electrophiles with alkenes and have explored the use of alkylaluminum compounds as Lewis acids. They have employed these reactions to produce intermediates for the synthesis of corticosteroids, the hydroazulene skeleton of pseudoguaianolide antitumor agents, and the antibiotic pseudomonic acid.

One approach has been the use of intramolecular [2 + 2] cycloadditions of ketenes and keteniminium salts to construct complex polycyclic compounds. They found that the regiochemistry of the cycloaddition is controlled by electronic effects of the substituents on the double bond, rather than steric effects. Bicyclo [3.1.1]heptan-6-ones are now readily available for the first time, via the conversions shown below. Dr. Snider has prepared furofuranone, 1, which is closely related to the carcinogenic aflatoxins, in 3 steps. The sesquiterpenes β-trans-bergamotene, 2, and β-ylangene, 3, have also been synthesized by very direct routes. Synthesis of the antitumor agent lemnalol, 4, from 3 is currently in progress.



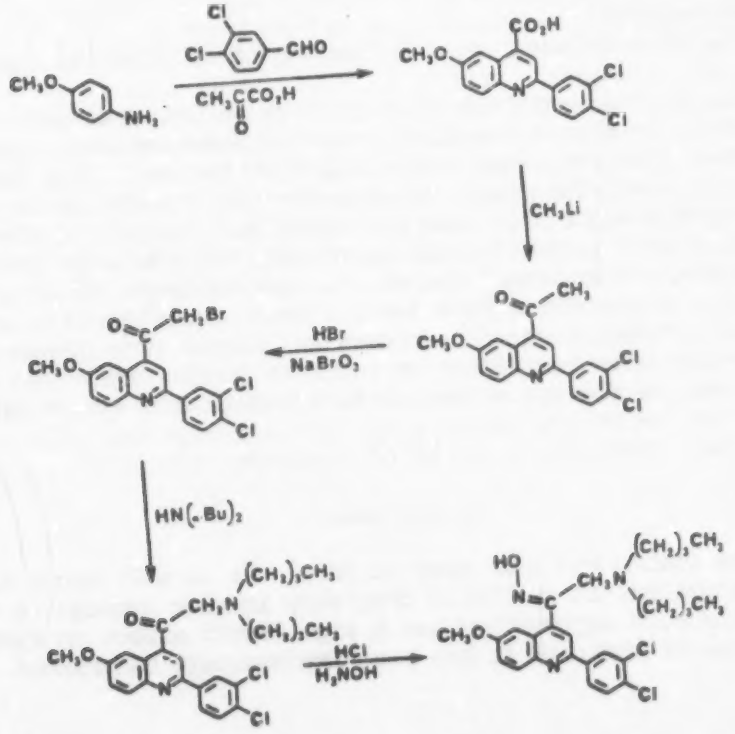


Chemical Model Reactions and Drug Design

"Superior Chemotherapeutic Agents by Rational Design"
R01 GM 33867-01 (Epling, G.), University of Connecticut

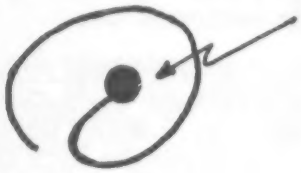
There is a clear need for new antimalarial compounds because of rapidly-developing resistance to currently used drugs. Some experimental materials that are structurally similar to quinine exhibit high levels of activity in laboratory tests, but side effects have precluded their medicinal use.

Dr. Gary Epling has focused his research upon careful alteration of the structure of highly active compounds in an attempt to eliminate the side effects while retaining their excellent pharmacological activity. Molecular orbital studies have suggested that attachment of an oxime functionality onto the most active compounds might make them retain their antimalarial activity, while the resulting change in hybridization would be expected to eliminate the side effects. One such compound has been synthesized, using the sequence given below. Preliminary studies of the ultraviolet/visible absorption spectra and fluorescence spectra of the product oxime show a significant change when DNA is added to the solutions, which probably indicates intercalation of the molecule into the DNA. This is exactly the behavior that was hoped for, as it was previously postulated that the antimalarial activity of compounds like quinine is due to their ability to intercalate DNA. Further studies will be needed to assess the possible utility of the new compound against malaria.



"Synthesis of Interesting Bio-Organic Molecules"
R01 GM 21457-08 (Menger, F.), Emory University

Dr. Fredric Menger has observed that long-chain esters hydrolyze in water much mo slowly than short-chain esters. This was interpreted to be due to a coiling of the chain about the ester group (indicated below as a black circle), thereby protecting the ester group from attack.



When an organic solvent such as ethanol is added to the water, the rate of reaction of the ester rises dramatically. Dr. Menger and his associates have extended these studies by measuring the effect of adding compounds of the general structure $R\text{-}NMe_3^+ \bar{I}$ to the long-chain ester in water, the "R" portion of the molecule being of 20 different groups. The compounds seem to induce the long-chain esters to unwind and thus expose the ester groups to reaction in the water. Different solvents manifest different rate increases; this presumably is related to how efficiently they interact with the coil. Dr. Menger feels that the increase in the rate of hydrolysis corresponds to hydrophobic interaction of the compound with the coil. Thus, he may have a sensitive and quantitative measure of group "hydrophobicity."

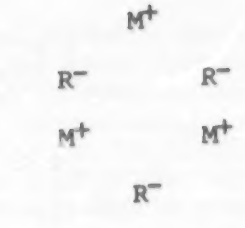
"Ion Pair Carbon Acidity"
R01 GM 30369-03 (Streitwieser, A.), University of California, Berkeley

An important step in the synthesis of many drugs and natural products is the removal of a proton to form a negatively charged intermediate, usually in close association with a positive metal such as lithium cation. This "ion pair" intermediate is then treated with other reagents to complete the synthetic step. In order that the synthetic organic chemist may better design a synthetic sequence, Dr. Andrew Streitwieser is determining quantitatively the relative stabilities of such negatively charged intermediates within various types of ion pairs. Measurements at different temperatures have led to the recognition of a novel rotational effect. In an important class of hydrocarbons, one or more groups that are relatively free to rotate are effectively frozen in the negatively charged intermediate. This loss of freedom of motion is a significant effect that makes the

$R^- M^+$

an ion pair

intermediate less stable and less easy to form. It is well known that many ion pairs that are important in synthetic chemistry involve aggregates of two or more units. While such aggregation has a significant effect on synthetic chemistry, what causes or controls it has been inadequately understood.

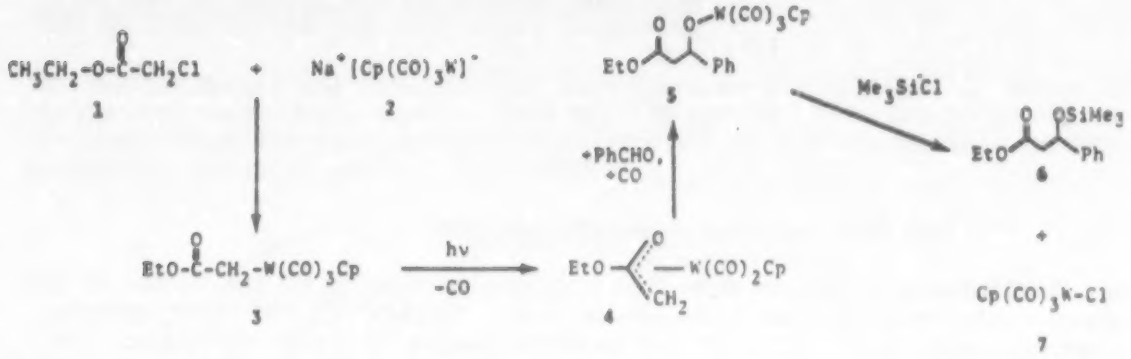


an aggregate (trimer)

Dr. Streitwieser's results show that aggregation depends on the nature of the organic substrate, the metal cation, solvent, temperature and other species present in solution. The more the negative charge is spread throughout the organic substrate, the more the ion pair is likely to be monomeric—not aggregated. Conversely, the more the charge is concentrated, the more the system tends to form aggregates.

"Preparation, Characterization and New Aldol Chemistry of Organotransition Metal Enolate Complexes"
R01 GM 25459-07 (Bergman, R.), University of California, Berkeley

One of the most important and widely-utilized techniques available for making new carbon-carbon bonds in organic molecules is the aldol reaction. This is typically carried out between an electrophilic carbonyl compound, such as an aldehyde, and a lithium or sodium enolate. Based on the hypothesis that the chemistry of transition metal enolates will be both broader and more easily controlled than that of alkali metal enolates, Dr. Robert Bergman and his collaborators have initiated the preparation and study of organotransition metal enolate complexes. Their work has focused specifically on enolates of the so-called "later" transition metals (those in group VI and higher), since these metals should have the metal center bound to carbon, rather than to oxygen. They have now prepared several organotransition metal enolates and examined their reactions with organic electrophiles. They find these materials in general to be much easier to isolate and characterize than their lithium and sodium analogs, and bonds to the metal centers are much more covalent. The compounds undergo photochemically initiated aldol reactions with aldehydes. The chemistry of one of the systems investigated is summarized in the drawing below. Reaction of halocarbonyl compound 1 with the readily available anionic tungsten complex 2 gives the tungsten ester enolate 3, whose structure has been established by X-ray diffraction. Irradiation of 3 in the presence of benzaldehyde leads to the metal-complexed aldol condensation product 5, which can be converted to the silylated aldol 6 (and regenerated metal halide 7) by treatment with chlorotrimethylsilane. Careful monitoring of this reaction by NMR spectroscopy has provided evidence that the initial irradiation leads to η^3-oxaallyl intermediate 4, which can react thermally with aldehydes to give 5.

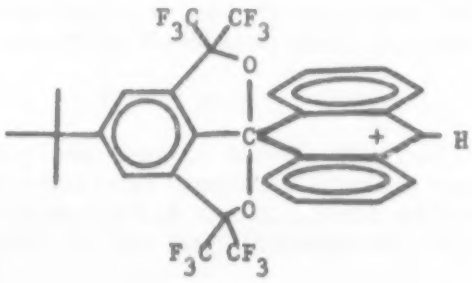


Further studies on the chemistry of transition state metal enolates is now under way, and preliminary experiments indicate that addition of the enolate fragment to alkynes is also possible. Thus, it appears that transition metal enolates are chemically analogous to sodium and lithium enolates. Furthermore, new reactions not now available to such main-group species will be accessible using their transition metal relatives.

"Synthetic and Physical Studies of Anticancer Agents"
R01 GM 33064-13 (Martin, J.), University of Illinois at Urbana-Champaign

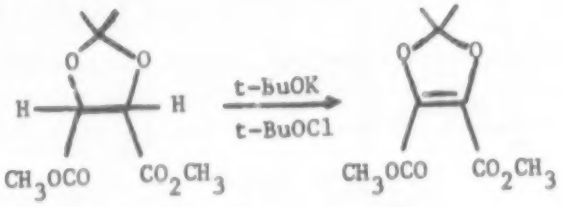
The fact that there are so many known organic compounds, many of biological importance, depends on the many ways in which networks of carbon-carbon bonds can be constituted to connect carbon atoms in valence states 2, 3, and 4. In addition, Dr. James Martin recently reported the synthesis and study of the first example of an observable pentavalent carbon species. Thus, it now appears that carbon valence states of 2, 3, 4, and 5 are available for the construction of organic compounds. Dr. Martin is extending his research toward the study of a relatively simple compound which he expects to contain pentavalent carbon and which should provide a better understanding of the features of molecular structure that are required for a stable pentavalent carbon center. He has preliminary evidence that he has synthesized compound 1, containing a pentavalent carbon.

Pentavalent carbon centers have long been postulated to occur in reaction transition states, but these are the first reports of stable, observable molecules.



Dr. Martin also has developed synthetic methods for preparing ligands designed to stabilize a variety of hypervalent species besides carbon, including non-metals such as boron, sulfur, phosphorus, selenium and tellurium. For example, he has developed conditions for replacing a somewhat acidic proton on an aromatic ring with lithium, by carrying out the lithiation in the presence of a trapping reagent that reacts with the aryllithium before it destroys itself by "in situ trapping". He has used this approach to prepare a series of hitherto inaccessible nitrogen heterocycles.

A mechanistically related reaction uses the strong base t-butoxide anion to remove a proton from carbon to give an anion, which reacts quickly with t-butyl hypochlorite to form a C-Cl bond. Subsequent rapid reaction of the chloride with the strong base in the same reaction medium introduces the desired double bond between two carbons. An example of this potentially widely useful synthetic method forms 2 in high yield.

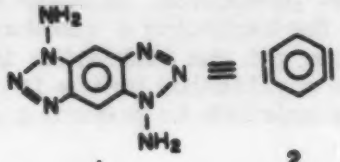


2

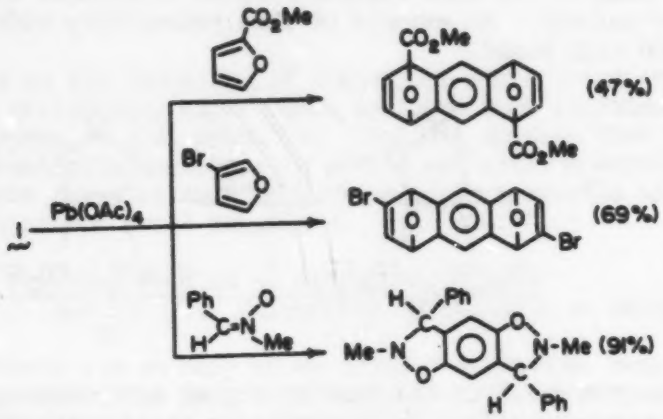
Thus, this research has led to the possibility of synthesizing unprecedented hypervalent species, as well as to the development of synthetic methodology likely to be of wide applicability.

"Studies in Organic Synthesis"
R01 GM 15997-15 (Hart, H.), Michigan State University

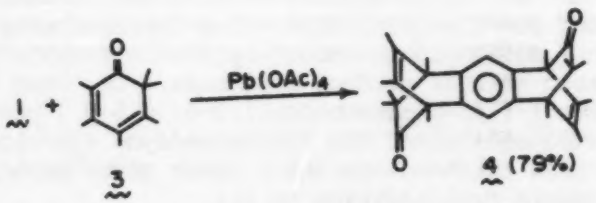
Dr. Harold Hart and his coworkers are seeking new, creative ways to use arynes in carbon-carbon bond construction, especially for synthesizing polycyclic aromatic compounds. He has developed two novel synthetic methods that could be broadly useful for aromatic natural product synthesis. Dr. Dong Ok synthesized the previously unknown 1 (1,5-diaminobenzo[1,2-d: 4,5-d] bistriazole), which can be used as a synthetic equivalent for 1,4-benzadiyne, 2. For example, treatment of compound 1 with lead tetraacetate and a diene gives good yields of bis-adducts (equivalent to double cycloaddition to 2):



1 2



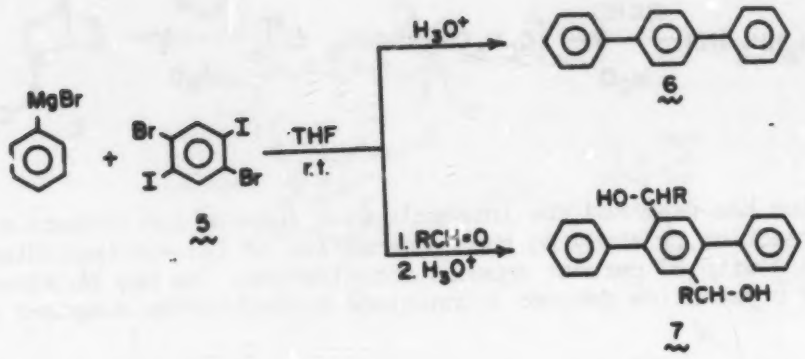
As seen from the product structures, the reaction shows remarkable regioselectivity. One example is the cycloaddition to 3, which gives mainly one of four possible regio- and stereoisomeric products, 4 (structure by single crystal X-ray).



3

Compound 1 is useful for fusing two new rings onto an existing benzene ring. Similar linear multi-ring arrangements are found among anthracycline antibiotics, so new materials obtained via compound 1 are being tested for biological activity.

K. Harada and C.-J. Frank Du have found a new way to use the addition of organometallics to arynes for constructing two to six new carbon-carbon bonds in a 'one-pot' reaction. The method involves reaction of polyhalogenated aromatics with excess Grignard reagents. Examples are the synthesis of 6 (2 new C-C bonds) and 7 (4 new C-C bonds) from 5 and excess phenylmagnesium bromide:



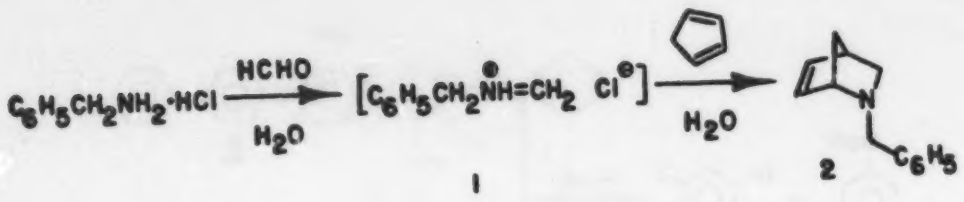
This chemistry has vast synthetic potential, as aryl, heterocyclic, vinyl and many other Grignard reagents or other carbanions may be used with a variety of polyhaloarenes.

"The Intramolecular Cation Radical Diels-Alder Reaction"
RO1 GM 34281-01 (Bauld, N.), University of Texas, Austin

Catalytic versions of organic reactions are important because they save energy and also are often more selective than non-catalytic versions. This results in more efficient production of desired, useful products, with lesser production of unwanted by-products. Dr. Nathan Bauld has been employing cation radicals to catalyze intramolecular Diels-Alder reactions. In this procedure, known as hole- or cation radical-catalysis, a few percent of a stable cation radical are used as catalyst, and organic molecules have been induced to undergo the intramolecular Diels-Alder in virtually quantitative yield in less than ten minutes. In the absence of the catalyst, the molecules are stable indefinitely. These cation radical-induced reactions are not only rapid and efficient but also are highly selective and stereospecific. When the full scope of these reactions is clarified and optimum procedures determined, they should dramatically facilitate the stereospecific syntheses of a wide variety of natural product and health-related organic molecules.

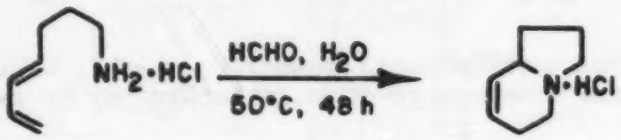
"Bio-Active Compounds via Aqueous Diels-Alder Chemistry"
RO1 GM 33605-05 (Grieco, P.), Indiana University

Dr. Paul Grieco and his colleagues have developed an imino variation of the all-carbon Diels-Alder reaction. The new aza Diels-Alder reaction, which features cyclocondensation of dienes with simple iminium salts generated under Mannich conditions, proceeds in water under exceptionally mild conditions. They have focused their preliminary studies on the reaction of cyclopentadiene with benzyl iminium ion 1 derived from formaldehyde and benzylamine hydrochloride. Addition of neat cyclopentadiene to a solution of benzylamine hydrochloride and formaldehyde in water at ambient temperature gives rise to a near quantitative yield of bicyclic amine 2. The procedure is applicable to a number of other dienes and amines.



1 2

Dr. Grieco also has examined the intramolecular iminium ion variation of the Diels-Alder reaction by studying the condensation of (E)-4,6-heptadienylamine hydrochloride 3 with 37 percent aqueous formaldehyde. He has obtained a 95 percent yield of crystalline dehydro δ-coniciene hydrochloride compound 4.



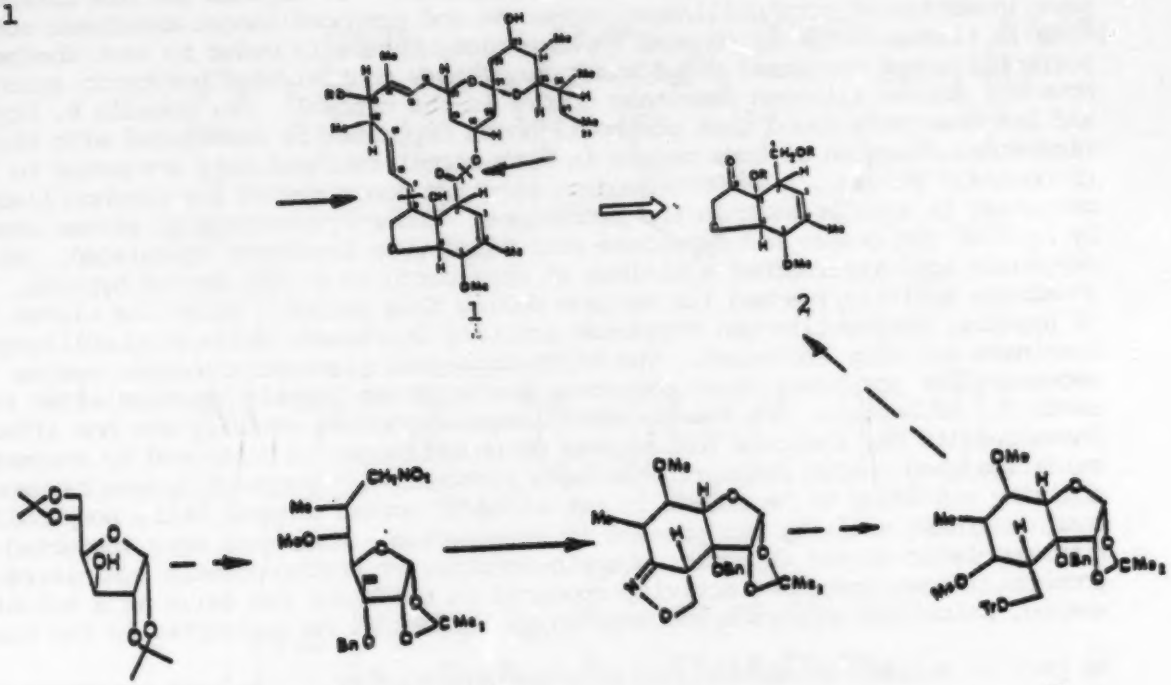
3 4

Information from Dr. Grieco's research should be helpful in developing procedures for the total synthesis of biologically important alkaloids, steroids, and terpenes whose potential as therapeutic agents range from antitumor to antibiotic activity.

"Trichothecanes, Amino Sugars, and Avermectins"
R01 GM 32569-02 (Fraser-Reid, B.), Duke University

The objective of Dr. Bertram Fraser-Reid's research is to explore new methods of employing D-glucose and its readily available derivatives for the synthesis of trichothecanes, amino sugars, and avermectins. He is pursuing strategies designed to take maximum advantage of the chemical and physical properties that the sugars offer.

Scheme 1



Avermectin, 1, can be dissected at the points indicated by the arrows, to give "northern" and "southern" segments. Dr. Fraser-Reid has synthesized the "southern" segment represented by 2. The starting material was a commercially available derivative of D-glucose, known commonly as diacetone glucose, 3. The sequence summarized in Scheme 1 features intramolecular cycloaddition of 4, which gives only the desired C2 isomer, 5. Standard processing then led to 6, from which 2 was obtained in excellent yield. The key step in this procedure is the conversion of 4 into 5. Dr. Fraser-Reid finds that this conversion is tolerant of a variety of structural types. Hence, it should be possible eventually to prepare several analogs of avermectin itself for biological evaluation.

Biosynthesis

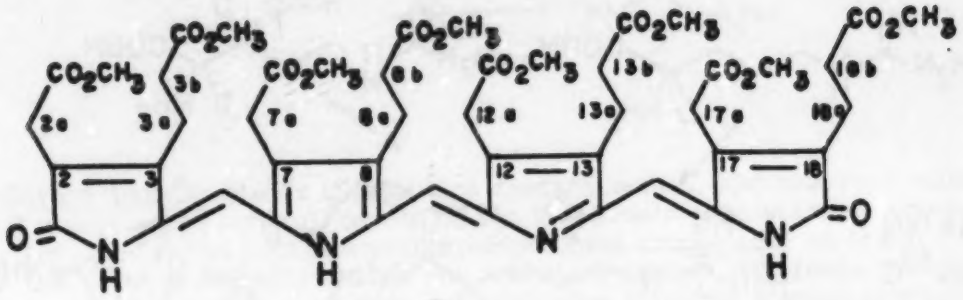
"Biosynthetic Intermediates of Porphyrins"
R01 GM 11973-19 (Frydman, B.), University of Buenos Aires

Several years ago, Dr. Benjamin Frydman found that porphobilinogen is the substrate of two enzymes of heme metabolism: of porphobilinogen deaminase, which, in cooperation with uroporphyrinogen III cosynthase, converts it into the porphyrin precursor uroporphyrinogen III, and of porphobilinogen oxygenase, which oxidizes it to oxopyrrolinones. Enzymatic oxidation of porphobilinogen to

oxopyrrolines removes it from the heme biosynthetic pathway and thus could be important in the regulation of heme biosynthesis. Dr. Frydman and his colleagues have investigated porphobilinogen oxygenase and porphobilinogen deaminase activities in tissues actively engaged in heme biosynthesis in order to test whether porphobilinogen oxygenase might exert regulatory control over porphyrin synthesis. However, porphobilinogen deaminase occurs in the cytosol. Dr. Rosalia B. Frydman and her coworkers found that porphobilinogen oxygenase is associated with the microsomal fraction of bone marrow in both normal rats and rats subjected to erythropoietic stress. A NADPH-dependent electron donor system for porphobilinogen oxygenase is also present in the microsomes. Under erythropoietic stress caused by hypoxia, oxygenase and deaminase activities were inversely correlated. While oxygenase activity reached a minimum at the fourth to eighth day of hypoxia, deaminase activity reached its maximum during this period. After the eighth day of hypoxia, porphobilinogen oxygenase activity increased, while porphobilinogen deaminase activity decreased. The NADPH-dependent electron transport system necessary for porphobilinogen oxygenase activity was largely inactive after the tenth day of hypoxia, but the porphobilinogen oxygenase activity was not affected. These results may indicate that excess porphobilinogen is destroyed by enzymatic oxidation when enough porphyrin has been synthetized. Porphobilinogen oxygenase activity was found to be absent in rat blood of normal animals while porphobilinogen deaminase activity was present in this system. When rats were subjected to erythropoietic stress (hypoxia, phenylhydrazine, or erythropoietin administration), porphobilinogen oxygenase activity appeared in the blood red cells as a soluble enzyme, coincident with the increase in the number of reticulocytes in the blood.

As part of related research concerning the specificity of the heme oxygenase-biliverdin reductase system, Dr. Frydman is preparing a series of synthetic biliverdins. This has allowed him to establish unambiguously the structure of bactobilin, a new bile pigment which was isolated recently from extracts of the anaerobic microorganisms Clostridium tetanomorphum and Propionibacterium shermanii. By comparing bactobilin with the synthetic urobiliverdin isomers using ^{1}H-NMR and HPLC procedures, Dr. Frydman made the surprising and unexpected finding that bactobilin, from the bacteria, is identical to urobiliverdin I (I as acid, Chart I).

Chart I : The urobiliverdin isomers



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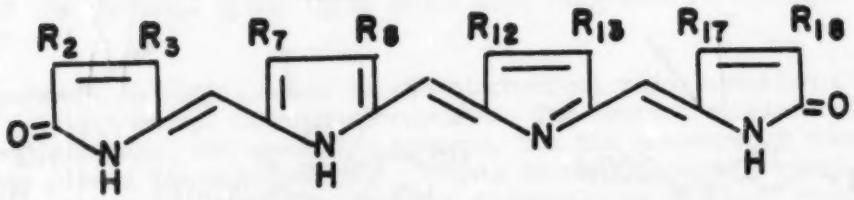


III α $\begin{cases} R_2 = R_7 = R_{13} = R_{17} = CH_2CO_2CH_3 \\ R_3 = R_8 = R_{12} = R_{18} = CH_2CH_2CO_2CH_3 \end{cases}$

III β $\begin{cases} R_2 = R_8 = R_{12} = R_{17} = CH_2CO_2CH_3 \\ R_3 = R_7 = R_{13} = R_{18} = CH_2CH_2CO_2CH_3 \end{cases}$

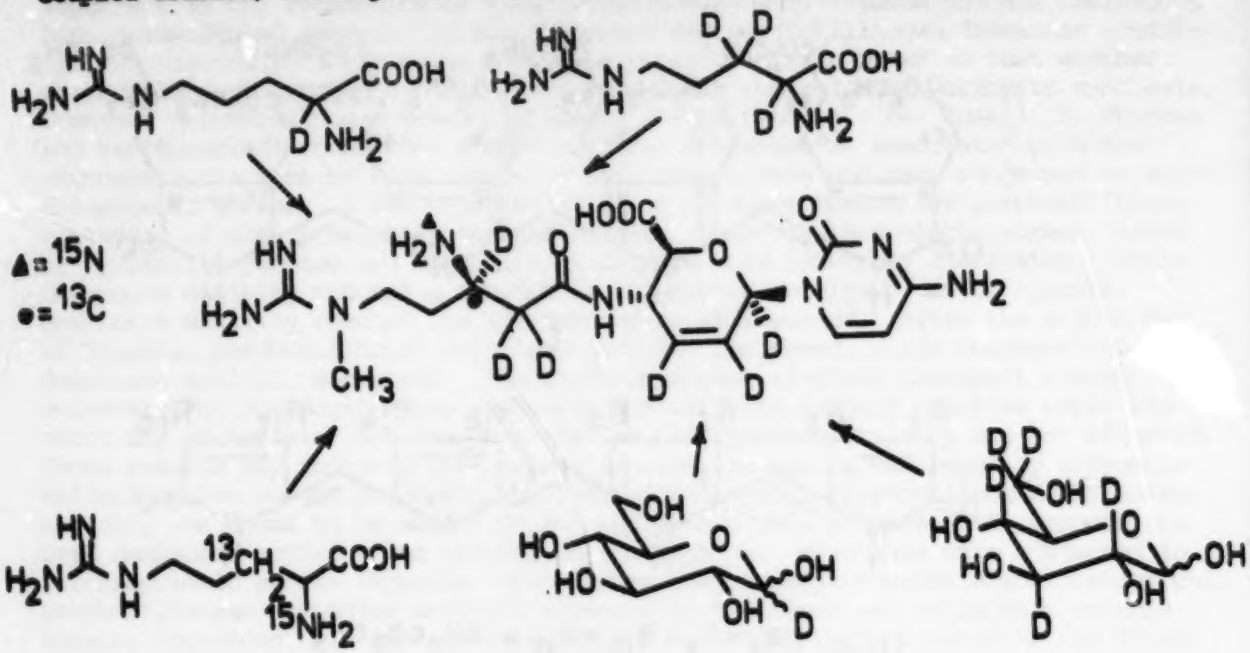
III γ $\begin{cases} R_3 = R_7 = R_{12} = R_{17} = CH_2CO_2CH_3 \\ R_2 = R_8 = R_{13} = R_{18} = CH_2CH_2CO_2CH_3 \end{cases}$

III δ $\begin{cases} R_2 = R_7 = R_{12} = R_{18} = CH_2CO_2CH_3 \\ R_3 = R_8 = R_{13} = R_{17} = CH_2CH_2CO_2CH_3 \end{cases}$

"Biosynthetic Studies Using ^{15}N-^{13}C NMR Spectroscopy"
R01 GM 32110-03 (Gould, S.), Oregon State University

Dr. Steven Gould is using radioactive feeding studies, coupled to NMR analysis, to study the biosynthesis of streptothricin F, negamycin, AT-125, and blasticidin S. Feeding of [2-^{2}H]-α-Arginine yielded blasticidin S labeled with deuterium at C-12, but only 20 percent of the original deuterium label was retained. When [2,3,3-$^2H_3$[-α-arginine was fed, deuterium was found at C-12 and at C-13 in a ratio of 1.2 to 1. Deuterium probably was lost from C-2 of arginine via an arginine racemase; this possibility is being tested with [3,3-$^2H_2$]-α-arginine.

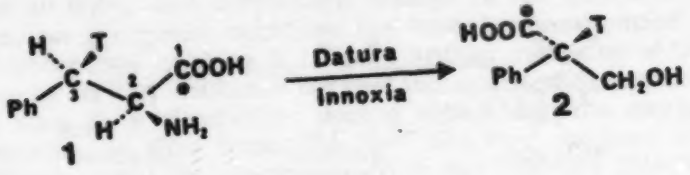
When [3-^{13}C,2-^{15}N]-α-arginine was fed, the ^{13}C NMR spectrum contained a spin-coupled doublet, showing retention of both labels and that the original α-amino



group had migrated intramolecularly. These results can be explained by the double rearrangement illustrated below.

"Metabolism of Natural Products of Medicinal Interest"
R01 GM 13246-28 (Leete, E.), University of Minnesota

(S)-Tropic acid, 2, is the acid moiety of the ester alkaloids hyoscyamine and scopolamine. It has previously been established that this acid is formed from phenylalanine, 1, with an intramolecular migration of the carboxyl group from C-2 to C-3. Dr. Edward Leete has investigated the fate of the hydrogens attached to C-3 of the phenylalanine side chain by labelling phenylalanine stereospecifically at this position with tritium. When (2S,3R)-[1-^{14}C,3-^{3}H] phenylalanine



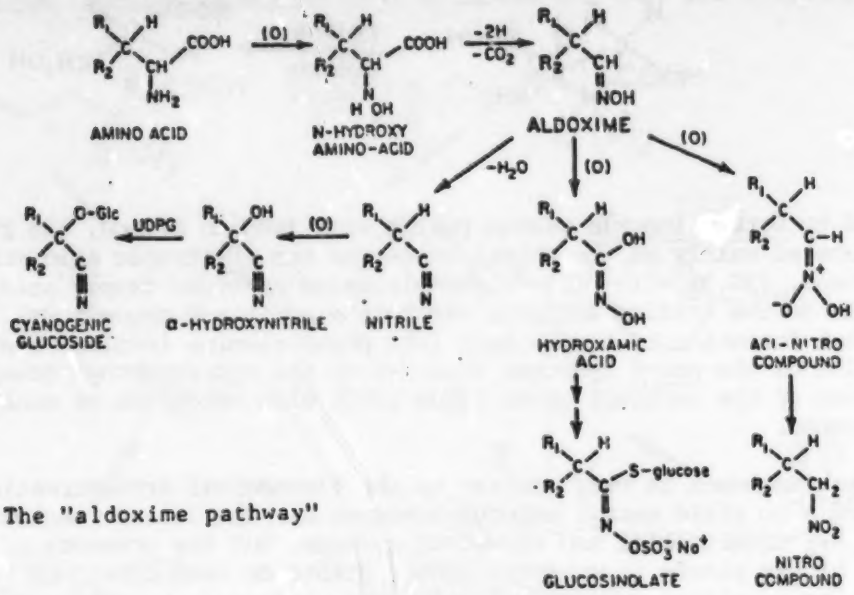
was fed to Datura innoxia plants by the wick feeding method, the resultant alkaloids were labeled mainly at the chiral center of the (S)-tropic acid moiety. On the other hand, (2S,3S)-[1-^{14}C,3-^{3}H]phenylalanine afforded tropic acid in which the bulk of the tritium activity was on the methylene group. Dr. Leete's results show that formation of tropic acid from phenylalanine involves a stereospecific migration of the pro-S hydrogen from C-3 to the hydroxymethyl group, and that migration of the carboxyl group takes place with retention of configuration as illustrated.

This rearrangement is very similar to the biochemical transformation of succinyl coenzyme A to yield methyl malonyl coenzyme A. The latter reaction utilizes coenzyme B_{12} in microbial and mammalian systems, but the presence of coenzyme B_{12} in higher plants is controversial. Plants do need cobalt to remain healthy, and B_{12} or a related cobalt derivative may ultimately be isolated from higher plants.

"Metabolism of Aromatic Compounds"
R01 GM 05301-28 (Conn, E.), University of California, Davis

Many higher plants convert the aromatic amino acids phenylalanine and tyrosine to cyanogenic glycosides by the biosynthetic pathway shown below. This pathway also accounts for the conversion in many other higher plants of the aliphatic amino acids valine, isoleucine and leucine to cyanogenic glycosides. (Some animals, including millipedes and zygaenid moths, also utilize the pathway to produce cyanogens.) While studying the formation of an unknown cyanogenic compound from tyrosine in cell suspension cultures of California poppy (Eschscholtzia californica), Dr. Eric Conn and his colleagues observed the formation of 1-(4'-hydroxyphenyl)-2-nitroethane. This nitrocompound is produced by cells which are osmotically stressed by 0.5 M sorbitol, a technique which is known to stimulate the production of isoquinoline alkaloids by the same cells. Microsomal preparations from stressed cells catalyze the conversion of either L-tyrosine or 4-hydroxyphenylacetaldoxime to 1-(4'-hydroxyphenyl)-2-nitroethane in the presence of NADPH. The aci-nitrocompound, produced by N-hydroxylation of the aldoxime, is tentatively postulated as an intermediate in the formation of 1-(4'-hydroxyphenyl)-2-nitroethane.

Thus, a third class of toxic natural plant product--compounds containing an aliphatic nitro group--shares with cyanogenic glycosides and glucosinolates the initial steps of a biosynthetic pathway involving N-hydroxylation of amino acids. This class includes β-nitropropionic acid, which occurs as a glucose ester in two genera of legumes (Astragalus and Indigophera) and is responsible for the toxicity of several species to livestock.



The "aldoxime pathway"

Enzymes

"Experimental and Clinical Pharmacogenetics"
P01 GM 27028-05 (Weber, W.), University of Michigan at Ann Arbor

N-Acetylation by the enzyme acetyl-CoA:arylamine N-acetyltransferase (E.C 2.3.1.5) is important in the biotransformation of arylamines, hydrazines and biogenic amines of aromatic acids. Dr. Wendell Weber has designed a new, faster, and less problematic procedure for measuring the reaction rate of this enzyme. The new assay is characterized by two features: 1) arylamine N-acetyltransferase activity is measured by the disappearance of the arylamine as reflected by decreasing Schiff's base formation, a well-characterized, rapid, one-step reaction resulting in a stable adduct and 2) during the assay, the product, coenzyme A, is recycled by the acetyl phosphate/phosphoacetylase system or by carnitine O-acetyltransferase to the substrate, acetyl-CoA. This avoids the strong product inhibition that increased concentrations of coenzyme A would cause. This recycling of coenzyme A makes it possible to measure enzyme reaction rates at low but relatively constant acetyl-CoA levels that approximate those that occur in vivo.

"Magnetic and Modeling Studies of Cytochrome c Oxidase"
R01 GM 28451-03 (Wilson, L.), Rice University

Cytochrome c oxidase is a terminal enzyme in the electron-transfer chain of aerobic respiration in various life forms, including man. The chemical reaction catalyzed by this enzyme is a critical one, involving the reduction of atmospheric oxygen to water with the release of energy. Without an efficient catalyst such as cytochrome oxidase, the reduction of O_2 to H_2O is not completed but

usually terminates at H_2O_2, and sufficient energy is not produced for cellular activity. To date, no synthetic catalyst has been produced which can compete effectively with cytochrome oxidase activity. After reacting with oxygen, cytochrome oxidase is in its oxidized or resting state and contains iron (Fe^{3+}) and copper (Cu^{2+}) ions at a bimetallic active site where the oxygen-to-water reaction has occurred. In this form, the Fe^{3+} and Cu^{2+} centers are paramagnetic in that they both have unpaired electrons, with Fe^{3+} having five unpaired electrons and Cu^{2+} one unpaired electron. Dr. Lon Wilson's recent magnetic measurements performed at 20-200°K have reaffirmed his earlier results that the Fe^{3+} and Cu^{2+} ions in resting cytochrome oxidase are in strong "magnetic communication" (antiferromagnetic with $-J > 200\ cm^{-1}$), through a biochemical bridge to give a spin-coupled [Fe^{3+}-(bridge)-Cu^{2+}] site structure with only four unpaired electrons. In an attempt to identify the nature of the bridge, Dr. Wilson performed magnetic measurements on cyanide derivatives of resting cytochrome oxidase. The results show a greatly diminished Fe^{3+}-Cu^{2+} interaction ($-J \approx 30\ cm^{-1}$), consistent with replacement of the normal bridge in the resting enzyme by cyanide to give a new bridged structure [$Fe^{3+}(CN)Cu^{2+}$]. The much larger -J value for normal resting cytochrome oxidase implies that it has a single atom bridge, rather than a two atom bridge that might occur in [$Fe^{3+}(CN)Cu^{2+}$]. This, in turn, supports a catalytic mechanism with an O_2-bridged intermediate and a [Fe^{3+}-O-Cu^{2+}] resting state structure (Figure A). This result seems to rule out imidazole as a bridge, but other potential bridges, such as HCO_2^-, F^-, H_2O_2 still need to be examined.

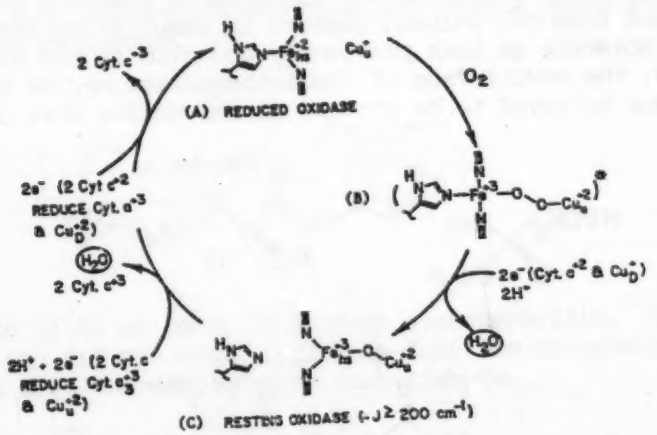
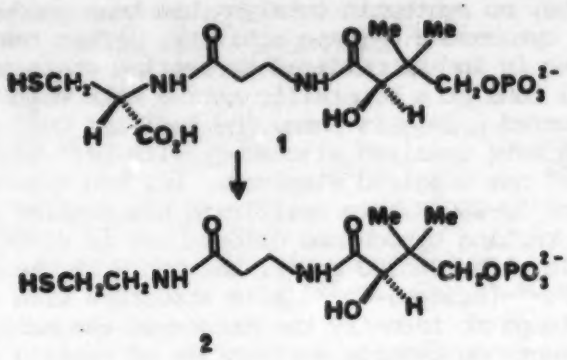


Fig. A

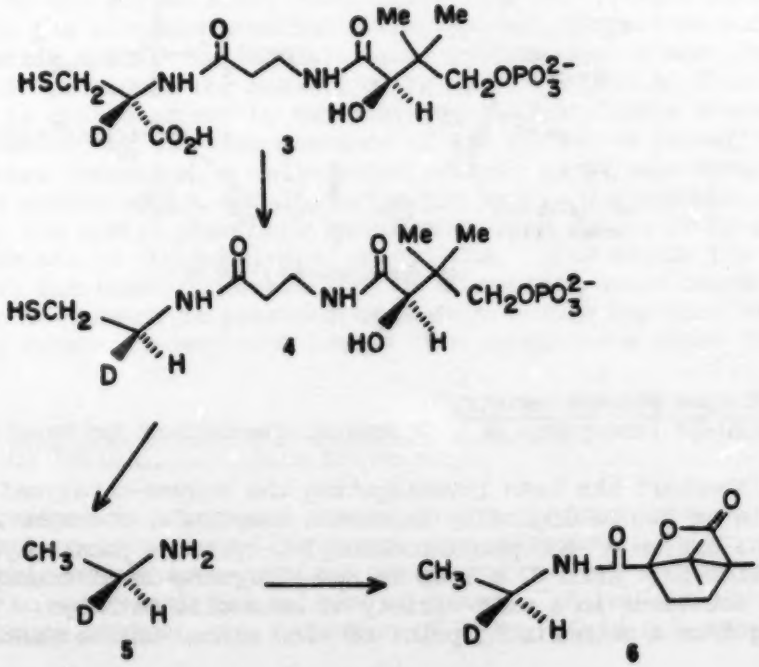
"Study on Enzyme Stereochemistry"
R01 GM 24420-07 (Aberhart, D.), Worcester Foundation for Experimental Biology

Dr. Donald Aberhart has been investigating the enzyme-catalyzed reactions in the biosynthesis of two biologically important compounds, coenzyme A and tetrahydrofolate. The enzyme 4'-phosphopantothenoyl-L-cysteine decarboxylase catalyzes the conversion of 1 into 2, a step in the biosynthesis of coenzyme A which is an acyl group activator in a wide variety of metabolic pathways. The enzyme is interesting from a mechanistic point of view since, unlike most decarboxylases,

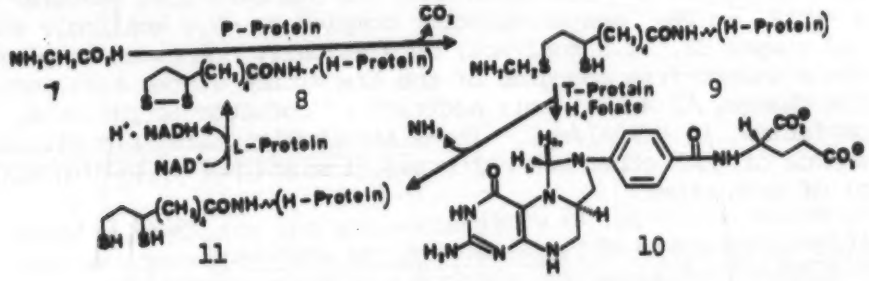


it apparently does not require any coenzyme, and there is no obvious mechanism for stabilizing the carbanionic intermediate that presumably is formed in the reaction. The enzyme appears to have an active site carbonyl group, the nature of which is still unknown.

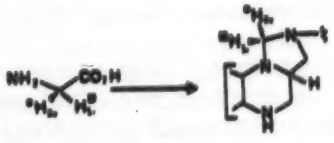
As part of an investigation of the mechanism of this enzyme, Dr. Aberhart determined the steric course of the decarboxylation. This was done by decarboxylating α-deuterated phosphopantothenoyl cysteine, 3, with rat liver phosphopantothenoyl cysteine decarboxylase. The product, 4, then was degraded to ethylamine, 5, which was converted to 6. The NMR spectrum of 6 showed that the incoming hydrogen atom, obtained from the solvent (water) is acquired by the substrate on the same face of the molecule as that previously occupied by the eliminated carboxyl group. Thus, the remote face of the intermediate may be blocked, possibly by a lysine residue believed to be present at the active site of the molecule.



Catabolism of glycine via the pathway shown below (7-11) constitutes one of the principal biochemical sources of single-carbon units in a wide variety of organisms. In this process, glycine is cleaved to form carbon dioxide, ammonia, NADH, and N^5,N^{10}-methylenetetrahydrofolate (5,10-CH_2-H_4folate). Both C-2 hydrogens of glycine are retained in the overall transfer of the methylene group from glycine to tetrahydrofolate in the formation of 5,10-CH_2-H_4folate.



Dr. Abernart has used stereospecifically deuterated glycine to determine the steric course of the two-step conversion of glycine to 5,10-CH_2-H_4folate in E. coli. He determined the stereochemistry of the labeled intermediate, 5,10-CH_2-H_4folate, by coupling its production (in vivo) with ketopantoate hydroxymethyltransferase, a 5,10-CH_2-H_4folate-dependent enzyme whose steric course he determined to be the following:



The process appears to be at least 78 percent stereospecific. The fact that complete racemization did not occur indicates that the methylene group transfer does not occur via free non-enzyme bound formaldehyde.

"Enzyme Effector Design"
R01 GM 20198-11 (Schowen, R.), University of Kansas

The enzyme pyruvate decarboxylase is of interest because it catalyzes a key reaction in carbohydrate metabolism, it uses thiamin pyrophosphate as a catalytic cofactor, and it undergoes covalent regulation of its enzyme activity (one molecule of pyruvate serves as a covalent activator while a second undergoes decarboxylation). Dr. Richard Schowen has examined the isotope effect of ^{13}C-labeled and deuterated substrates during the pyruvate carboxylase reaction in order to elucidate the reaction mechanism. He finds that the carboxyl-C-13 effect for Vmax is 2.5 percent, which is half the value for decarboxylation. This indicates that the decarboxylation event is half-rate-limiting and has an intrinsic rate constant of 2 kcat. The beta secondary-D isotope effect shows

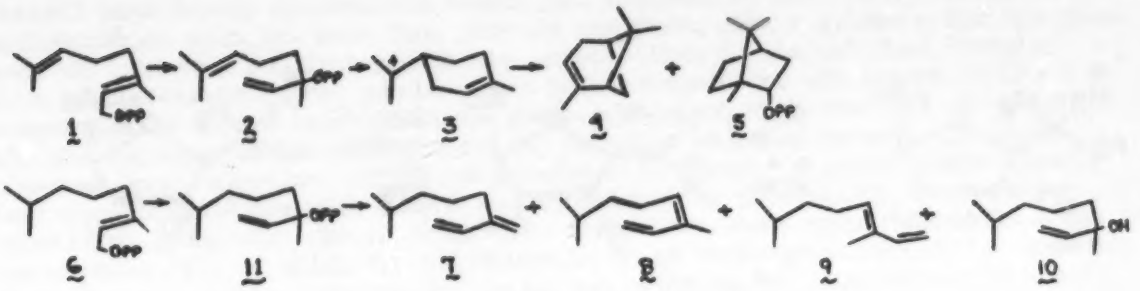
that the other contributing step to Vmax involves carbonyl elimination. Decomposition of the acetaldehyde-thiamin pyrophosphate adduct and product release also has a rate constant of 2 kcat. Solvent isotope effects confirm previous hypotheses that the regulatory event is the addition of an enzymic sulfhydryl group to pyruvate. The 2-^{13}C isotope effect and the small 1-^{13}C effect on kinetic terms other than Vmax show that binding of the non-regulatory pyruvate largely determines the rate (85 percent) below saturation.

From these data, Dr. Schowen calculated the standard free energies of the intermediate states to be: enzyme:cofactor complex E, -2.8 kcal/mol; substrate-activated enzyme ES, -2.5 kcal/mol; catalytically competent complex, -2.8 kcal/mol. The standard free energies of the transition states are: activating conformation change, 12.4 kcal/mol; addition of cofactor to pyruvate, 12.7 kcal/mol; decarboxylation, 11.8 kcal/mol. Thus, all stable states lie within a span of 0.5 kcal/mol of each other and all transition states lie within a span of 1 kcal/mol of each other.

"Enzymatic Cyclization of Terpenoid Natural Products"
R01 GM 31354-03 (Croteau, R.), Washington State University

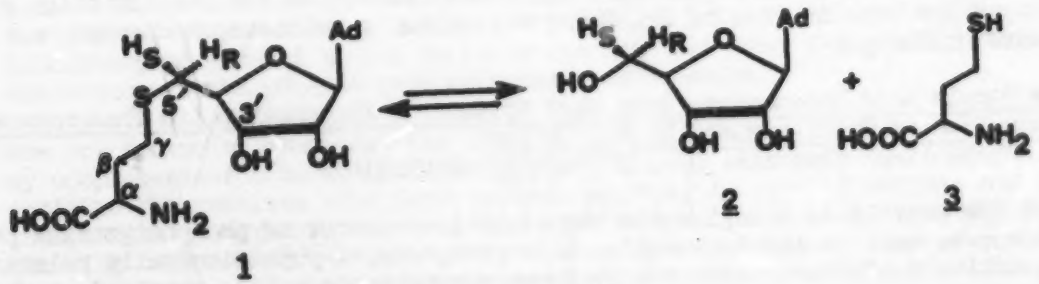
The cyclization of prenyl pyrophosphates represents an important mechanism of enzymatic C-C bond building and the route to a wide variety of natural products of pharmacological significance. The crucial cyclizations for generating monoterpene carbon skeletons are catalyzed by enzymes collectively known as cyclases. All known cyclases can convert geranyl pyrophosphate, 1, to cyclic product without the involvement of free intermediates and in spite of the fact this trans-allylic precursor cannot cyclize directly. These enzymes, therefore, are capable of catalyzing the isomerization of the precursor to a bound intermediate suitable for cyclization, as well as the cyclization itself.

Dr. Rodney Croteau has been studying simple monoterpene cyclizations as models for this general electrophilic reaction type. Cyclases from Salvia officinalis, capable of catalyzing the conversion of geranyl pyrophosphate to (+)-α-pinene, 4, and (+)-bornyl pyrophosphate, 5, were examined with the non-cyclizable substrate analog 6,7-dihydrogeranyl pyrophosphate, 6, in an attempt to dissect the normally cryptic isomerization step from the coupled reaction sequence. The analog inhibited cyclization of geranyl pyrophosphate but was itself catalytically active, affording as products the dihydro analogs of myrcene, 7, cis-ocimene, 8, trans-ocimene, 9, and linalool, 10. The biosynthetic products differ significantly from the simple solvolysis products of dihydrogeranyl pyrophosphate in that they comprise a far higher proportion of olefins. This suggests that enzymatic product formation occurs in an environment relatively inaccessible to solvent. The substrate analog seems to undergo the normal pyrophosphate migration step to 6,7-dihydrolinalyl pyrophosphate, 11, which, because the normal cyclizations are precluded, ionizes and is deprotonated to olefins, or is captured by water. Enzymatic transformation of the dihydro analog resembles the normal cyclase reaction in its divalent metal ion requirement, pH optimum and other characteristics, further suggesting that the catalytic activity observed represents the successful decoupling of the isomerization step from the cyclization step of the reaction sequence.



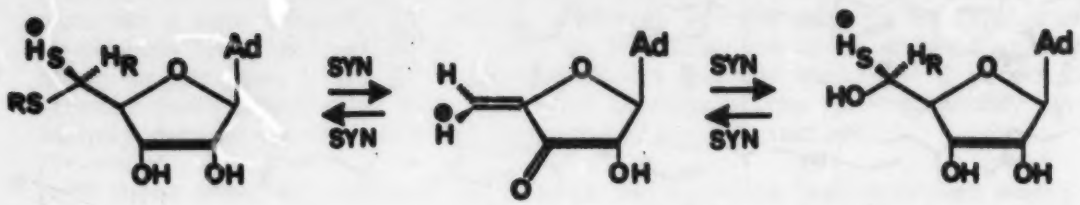
"Metabolism of Chirally Labeled Adenosine"
R01 GM 26166-07 (Parry, R.), Rice University

Dr. Ronald Parry is examining the stereochemistry of important enzymatic transformations that involve adenosine. One of these is the reaction catalyzed by S-adenosylhomocysteine hydrolase. The substrate for this enzyme is S-adenosylhomocysteine, 1, which is a product of biological transmethylation reactions that utilize S-adenosylmethionine as the methyl donor. S-adenosylhomocysteine is a potent inhibitor of most methyltransferases and it may play a regulatory role in vivo. The only known mechanism for the catabolism of S-adenosylhomocysteine in eukaryotic cells is via its reversible hydrolysis to adenosine, 2, and homocysteine, 3, catalyzed by S-adenosylhomocysteine hydrolase (Equation 1). This enzyme was first isolated from rat liver and subsequently was found in a variety of eukaryotes. The enzyme from beef liver has been crystallized and characterized. These studies established that the enzyme contains bound NAD and that cleavage of S-adenosylhomocysteine to homocysteine and adenosine is initiated by oxidation of the 3' hydroxyl group of S-adenosylhomocysteine, followed by β-elimination of homocysteine to yield 3'-keto-4',5'-dehydro-5'-deoxyadenosine. The latter substance then undergoes addition of water to produce 3'-ketoadenosine, which is then reduced to adenosine. Dr. Parry has performed a complete stereochemical analysis of this reaction sequence by incubating stereospecifically deuterated forms of the substrate or of 4',5'-dehydro-5'-deoxyadenosine, which also serves as a substrate, with the enzyme and determining the chirality of the deuterium labels in the products with nuclear magnetic resonance spectrometry. The results (Equation 2) show that the enzyme catalyzes a syn elimination of homocysteine, followed by a syn addition of water. This suggests a single base mechanism; the stereochemical path is known to be followed by enzymes that catalyze β-replacement reactions of α-amino acids via an elimination-addition sequence.

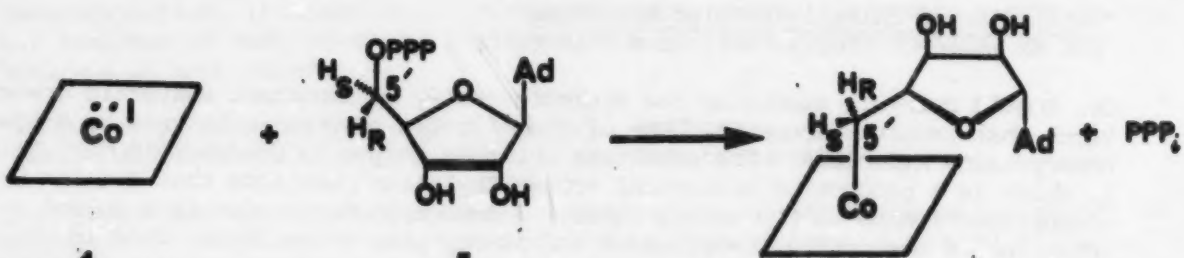


1 2 3

Equation 1



Equation 2



Equation 3

Dr. Parry's group also has elucidated the stereochemistry of the reaction catalyzed by cob(I)alamin adenosyltransferase from Clostridium tetanomorphum, an anaerobic bacterium. This enzyme, a form of which also occurs in mammalian cells, catalyzes the formation of coenzyme B_{12}, 6, from reduced vitamin B_{12}, (4), and ATP, 5, (Equation 3). The stereochemical analysis was carried out by incubating ATP that had been chirally deuterated at C-5' with the enzyme, isolating the resulting labeled coenzyme B_{12}, and determining its chirality at C-5' by nmr spectrometry. The results clearly showed that the carbon-cobalt bond in coenzyme B_{12} is formed with overall inversion of configuration, which is the same stereochemical result which had been observed earlier by Dr. Parry's group for the only other known adenosyltransferase, L-methionine s-adenosyl-transferase. Some previously reported experiments with Clostridium cob(I)alamin adenosyltransferase had suggested a multi-step mechanism for the formation of coenzyme B_{12} but, in view of Dr. Parry's results, a multistep mechanism now appears unlikely.

"The Purple Acid Phosphatase from Beef Spleen: A Phosphotyrosine Phosphatase Using Iron in Catalysis and in Regulation"
RO1 GM 32117-03 (Averill, B.), University of Virginia

Dr. Bruce Averill is studying the binuclear iron center of phosphotyrosine phosphatase, as well as its interaction with phosphate, a physiologically relevant competitive inhibitor. Iron K-edge X-ray absorption spectra, obtained at the

Cornell High Energy Synchrotron Source and analyzed at Bell Laboratories in collaboration with Dr. Boon Teo, provide the first direct evidence for the presence of iron-iron and iron-phosphorus interactions in the oxidized (purple) form of the enzyme. They suggest that both interactions are absent in the enzymatically active (pink) reduced form, consistent with the loss of phosphate and the decrease in the strength of the magnetic coupling between the atoms observed upon reduction. Electron paramagnetic resonance spectra show clearly that two forms of the pink enzyme exist, related by protonation of an as yet identified group with pKa of about 4.5. Resonance Raman spectra show at least seven peaks, all of which are attributable to an iron-tyrosinate unit. However, no evidence could be obtained for an oxygen bridging the iron atoms, due to photoreduction of the chromophore at the short wavelengths used.

Phosphate binding to the reduced (pink) enzyme causes oxidation of the binuclear iron center and identical time dependences for the optical spectral shift, loss of EPR signal, and loss of enzymatic activity. These results differ from those reported previously for the apparently similar porcine uterine enzyme (uteroferrin). Direct chemical analysis and radiochemical methods show that the reduced enzyme binds one mole of phosphate upon oxidation. Thus, active and inactive forms of purple acid phosphatase seem to be related both by an oxidation-reduction reaction and by phosphate binding, making it possible to regulate the enzyme activity in vivo by the intracellular redox potential as well as by phosphate levels.

L-Phosphotyrosine is the best substrate (K_m approximately 0.4 mM) for the enzyme to date. This suggests that purple acid phosphatase in vivo could function as a phosphotyrosine-specific phosphoprotein phosphatase, thereby regulating other as yet unidentified enzymes. The presence of the enzyme in spleen, the extreme variation in enzyme levels as a function of stress on the immune system of the cow, and the recently recognized importance of tyrosine phosphorylation as a key physiological control mechanism all suggest that purple acid phosphatase functions in concert with a tyrosine kinase to regulate the activity of other enzymes important in the immune response.

"Somatic Genetic Analysis of Adenylate Cyclase"
R01 GM 28310-05 (Bourne, H.), University of California, San Francisco

Dr. Henry Bourne's laboratory recently reported that guanine nucleotides regulate adenylate cyclase in membranes of the yeast S. cerevisiae, just as in vertebrate membranes. The catalytic unit (C) of the yeast enzyme, like that of vertebrates, requires stimulation by a G protein to utilize Mg-ATP as substrate but shows no such requirement with Mn-ATP as substrate. Yeast adenylate cyclase contains at least two components: C, which is thermally stable, and G_s, which is required for GTP-dependent activity and is thermolabile. G_s was shown recently by the laboratory of Michael Wigler to be encoded by the yeast RAS genes, which are homologous to the p21-ras proto-oncogenes of mammals.

Now Dr. Bourne's laboratory has isolated genomic DNA encoding the C component by complementation of a mutation (cyr-1-1) in the structural gene for C. The isolated DNA restores adenylate cyclase activity to cyr-1-1 mutants and directs integration at the CYR1 locus. Wild type strains transformed with CYR1 DNA on the high copy number vector YEp24 contain four- to six-fold more adenylate cyclase activity than strains carrying the plasmid with no insert. The increased

activity is observed with Mn-ATP as substrate and also when adenylate cyclase is stimulated by guanine nucleotides with Mg-ATP as substrate. This result suggests that expression of C, rather than that of other polypeptide components, including the RAS gene products, limits total adenylate cyclase activity in S. cerevisiae.

The similar regulation of adenylate cyclase from vertebrates and yeast suggests that isolation and characterization of the gene encoding yeast C will open avenues to elucidation of the structure and function of this important effector enzyme in vertebrates, as well as in yeast.

"Mechanisms of Catalysis by Glutathione S-Transferases"
R01 GM 31462-03 (Abdel-Monem, M.), University of Minnesota
"Detoxification of Xenobiotics in Erythrocytes"
R01 GM 32304-01 (Awasthi, Y. C.), University of Texas Medical Branch Galveston
"Factors in the Prevention of Toxic Liver Injury"
R01 GM 31555-02 (Boyer, T.), University of California, San Francisco

Many toxic xenobiotics appear to be either strong electrophiles or capable of producing strong electrophiles in the body. These electrophiles may react with critical body nucleophiles, leading to tissue damage. Conjugation with glutathione represents the body's first line of defense against toxic electrophiles. One way that the body deals with toxic nucleophiles is to convert them to a more water-soluble but less toxic form through the action of a family of enzymes known as the glutathione S-transferases.

Dr. Mahmoud Abdel-Monem is examining the stereochemical aspects and mechanisms of some conjugation reactions catalyzed by these important enzymes in order to delineate some of the structural features of foreign compounds that they recognize. He finds that conjugation of the alkyl and aralkyl halides proceeds with inversion of configuration at the chiral carbon, suggesting direct displacement of the halide with glutathione. Inversion of configuration still occurs when the enantiomers of 2-iodooctane and phenethyl chloride are used as substrates and glutathione S-transferase preparations are used in which 1, 2 or 3 of the 4 sulfhydryl groups have been blocked. The enantiomers of both trans-β-methyl styrene oxide and cis-β-methyl styrene oxide are substrates for partially purified rat liver glutathione S-transferases. Each enantiomer gave only one glutathione conjugate. The enzymatic conjugation of the β-methyl styrene oxide enantiomers is regioselective, via attack of glutathione sulfur on the benzylic carbon and inversion of configuration at the site of attack. The enzyme exhibits a high degree of enantioselectivity towards its various substrates; the enantiomer with R configuration at the benzylic carbon is metabolized much faster than the one with the S configuration, but S enantiomers of the phenethyl halides are metabolized faster than R enantiomers. However, glutathione S-transferases from rat liver do not show substrate enantioselectivity towards the isomers of 2-bromooctane and 2-iodooctane. These results led Dr. Abdel-Monem to conclude that minor structural differences between drugs and environmental contaminants may result in significant differences in their toxicity.

Dr. Yogesh Awasthi recently identified a new form of glutathione S-transferase in human erythrocytes that has kinetic and structural features which are different from all the other known human glutathione S-transferases. This new enzyme accounts for only 2 percent of enzyme activity of erythrocytes towards 1-chloro-2,4-dinitrobenzene as substrate, but it accounts for about 10 percent of total glutathione

S-transferase protein. The other human glutathione S-transferases of human tissues reported so far arise from different binary combinations of subunits having molecular weights in the range of 22,500 to 26,500. This new enzyme of erythrocytes is a heterodimer of subunits with molecular weights of 35,000 ± 3,000 and 14,000 ± 1,500 and is immunologically distinct from the known glutathione S-transferases of human erythrocytes, liver, placenta, and lung. Unlike other human glutathione S-transferases, this enzyme undergoes a transient activation upon incubation with sulfhydryl blocking reagents. The enzyme binds endogenous hydrophobic ligands such as hematin, and xenobiotics such as bromosulfophthalein, with high affinity; epoxide substrates such as 1,2-epoxy-3-(p-nitrophenoxy)-propane are preferred substrates. Along with his studies of the physiological role of this new form of glutathione S-transferase in the detoxification of xenobiotics and endogenously generated toxic compounds within erythrocytes, Dr. Awasthi also is examining the possibility that endogenous epoxides generated during lipid peroxidation in erythrocytes may be conjugated by this enzyme to glutathione.

Catalyzing a reaction between reduced glutathione and a variety of these toxic electrophiles, the glutathione S-transferases bind a variety of compounds that they do not metabolize, known as nonsubstrate ligands, and may act as storage and/or intracellular transport proteins for molecules having limited solubility in water. Dr. Thomas Boyer and his colleagues have been investigating the relationships between structure and function and between catalysis and the binding of nonsubstrate ligands by the glutathione S-transferases. Alkaline forms (pI greater than 7.0) of glutathione S-transferase account for more than 90 percent of the transferase activity in most organs of the rat. These alkaline enzymes are composed of four different subunits termed Ya, Yb_1, Yb_2, Yc. In contrast, 50 percent of the transferase activity in rat testes is due to acidic enzymes (pI less than 7.0). Dr. Boyer and his colleagues purified the acidic enzymes from testes and obtained one form that accounted for more than 90 percent of the transferase activity in the acidic fraction. It was composed of a new subunit (termed Yt) that differed from the Ya, Yb, Yc subunits of the alkaline enzymes in molecular weight, amino acid content, and functional and immunologic properties. Enzymes containing the Yt subunit were also identified in lung and kidney. Thus, some tissues have different transferase isozymes with different substrate specificities, and hence may have varying degrees of resistance to toxic electrophiles.

Dr. Boyer found that glutathione S-transferases act simultaneously as enzymes and binding proteins at pH values greater than 7.0. However, a human cationic form of glutathione S-transferase was inhibited less by a variety of nonsubstrate ligands at pH 9.0 than at pH 6.0, due to a reduced affinity of the human enzyme for the nonsubstrate ligands at the higher pH. Furthermore, the human transferase had little activity at pH 7.0 in the presence of saturating concentrations of nonsubstrate ligands. Thus, the mechanism of the pH effect seems to be different for the human enzymes from that of the rat enzymes. Since the normal intracellular pH is near 7.0, the accumulation of nonsubstrate ligands, as occurs in cholestasis, may render the human liver susceptible to injury by compounds that are normally metabolized by the glutathione S-transferases.

Enzyme Inhibitors

"Inhibitors of Processing and Lysosomal Glycosidases"
R01 GM 31355-02 (Elbein, A.), University of Texas Health Science Center, San Antoni

N-linked glycoproteins are important membrane receptors for binding various key biological molecules such as insulin, acetylcholine and low density lipoprotein. While many of these receptors have complex oligosaccharides, the role of such oligosaccharides in glycoprotein or receptor function is unclear. Several plant alkaloids have been identified that inhibit different processing steps for the trimming or processing of N-linked oligosaccharides. For example, swainsonine inhibits mannosidase II in cells and causes the production of hybrid types of oligosaccharides, whereas castanospermine inhibits glucosidase I and gives rise to high-mannose structures. The 2-epimer of swainsonine has been synthesized chemically by Drs. Adams and Sharpless and found by Dr. Alan Elbein to inhibit α-glucosidase. Thus, the hydroxyl groups at carbons 1 and 2 of the indolizidine are analogous to the 2 and 3 hydroxyl groups of the sugars. Epimerizing the 2 hydroxyl group of swainsonine gives the glucose analog. In the presence of the 2-epimer of swainsonine, cells accumulated $Glc_3Man_{7-9}GlcNAc_2$ structures on their glycoproteins.

Another compound, 1,4-dideoxy-1,4-imino-D-mannitol, has been synthesized chemically by Dr. George Fleet of Oxford via a simple, straightforward route. In tests by Dr. Elbein of its ability to inhibit glycosidase and glycoprotein processing, the compound proved to be a potent inhibitor of jack bean and lysosomal α-mannosidase and also inhibited mannosidase I; in its presence, the formation of complex oligosaccharides is inhibited, and $Man_9GlcNac_2$-proteins result.

It should be possible to prepare other stereoisomers that would inhibit other glycosidases. Such compounds should be valuable tools for modifying oligosaccharide chains and determining their roles in glycoprotein function. Many lysosomal storage diseases are due to the lack of a specific glycosidase. Inhibitors of the type described here may make it possible to inhibit specific enzymes in animals and, therefore, set up animal models for a variety of lysosomal storage diseases such as Tay-Sachs or Fabrys diseases.

"Tissue Specific Inhibitors of Phosphodiesterase"
R01 GM 21220-09 (Wells, J.), Vanderbilt University

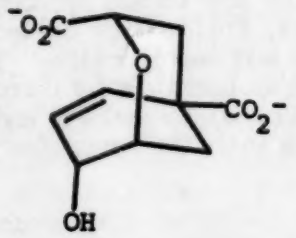
Although 4-azido-7-phenylpyrazole [1,5a]-1,3,5-triazine (APPT) is not a highly potent reversible inhibitor of calmodulin-dependent phosphodiesterase in the dark, Dr. Jack Wells found it to be an efficient irreversible inhibitor of this enzyme when exposed to light. In the dark, concentrations of APPT in excess of 100μm were needed in order to produce 50 percent reduction in hydrolysis of 1μM cyclic GMP. However, at much higher enzyme concentrations, incubation with 50μM APPT under photolytic conditions caused 70-80 percent inactivation of the enzyme. Photo-induced inactivation was time dependent (up to 30 seconds) and dependent on the concentraton of APPT. Inactivation appeared to be due to modification of the hydrolytic site, since it was attenuated by cyclic GMP and by potent, light-stable inhibitors such as 1-methyl-3-isobutylxanthine (100μM) and papaverine (100μM) but not by caffeine or theophylline. Thus, APPT may be a photoaffinity label for calmodulin-sensitive cyclic nucleotide phosphodiesterase. If so, isolation and identification of some of the peptide sequences involved in the binding of substrate and/or in the catalytic activity of the enzyme may be possible.

"Substrate Analogs for Terpene Condensation Reactions"
R01 GM 21328-11 (Poulter, C.), University of Utah

Isopentenyl pyrophosphate:dimethylallyl pyrophosphate isomerase catalyzes the interconversion of isopentenyl pyrophosphate and dimethylallyl pyrophosphate, an essential early step in the sterol biosynthetic pathway. However, very little is known about the isomerase or the reaction it catalyzes. As the first step toward the chemical characterization of its catalytic site, Dr. C. Dale Poulter has identified two classes of active-site-directed irreversible enzyme inhibitors. One class is a family of analogs of isopentenyl pyrophosphate and dimethylallyl pyrophosphate that have a fluorine allylic to the double bond. These compounds irreversibly inhibit isomerase activity and in the process become covalently attached to the enzyme. The inhibition constants are in the high nanomolar range and the inactivation rate constant is 0.3 minute $^{-1}$. The second class of inhibitors consists of ammonium analogs of isopentenyl pyrophosphate. These compounds have a positively charged nitrogen with attached methyl groups; preliminary data suggest that they transfer a methyl group to the enzyme during inactivation. Products of the sterol biosynthetic pathway are essential for survival in most, if not all, organisms. Thus, selective delivery of an irreversible inhibitor could have important applications in medicine and agriculture.

"Studies Related to Chorismic Acid"
R01 GM 28965-03 (Bartlett, P.), University of California, Berkeley

The transformation of shikimic acid to prephenic acid via chorismic acid is part of the pathway for the biosynthesis of aromatic amino acids in plants and microorganisms. In order to understand better the enzymic reactions of these unusual transformations and to investigate possible means of regulating them, Dr. Paul Bartlett has developed efficient laboratory syntheses of the intermediates along this pathway, including shikimic acid, 5-enolpyruvyl-shikimic acid, 3-phosphoshikimic acid, and 5-enolpyruvyl-3-phosphoshikimic acid. In addition, he has designed and synthesized several compounds as potential inhibitors of the chorismate mutases. The bicyclic diacid depicted below has proven to be the most potent inhibitor yet reported for the chorismate mutases. Potent inhibitors of the mutases could have practical importance, perhaps as selective herbicides.



"Mechanism and Inhibition of Diaminopimelate Metabolism"
R01 GM 39826-03 (Vederas, J.), University of Alberta

The diaminopimelate metabolic pathway, for the synthesis of the amino acid lysine, occurs in bacteria and higher plants but not in mammals. Mechanism-based irreversible inactivators (suicide substrates) of key enzymes which catalyze

the individual steps of the pathway might be useful as broad-spectrum antibiotics which are non-toxic to mammals. Dr. John Vederas is currently examining the mechanism and inhibition of two enzymes from late stages of this pathway: DL-diaminopimelate-D-dehydrogenase (E.C. 1.4.1.16) and DL-diaminopimelate decarboxylase (E.C. 4.1.1.20).

During studies on the mechanism and structural relationship of the decarboxylase from wheat germ and from bacteria (Bacillus sphaericus), he developed a new nuclear magnetic resonance (NMR) spectral method to determine the three dimensional arrangement of hydrogens isotopically substituted with deuterium. This general technique employs two dimensional NMR correlation of hydrogen and carbon-13 chemical shifts with deuterium decoupling and is especially useful in cases where there is considerable overlap of signals. Its application to deuterium-substituted L-lysine derivatives from the diaminopimelate decarboxylase reaction shows that both the bacterial and plant enzymes catalyze the transformation with an unusual inversion of configuration. Thus, these proteins have a close mechanistic relationship to each other and differ from most other enzymes which also use pyridoxal phosphate as a cofactor.

Dr. Vederas has synthesized and tested several diaminopimelate analogs for irreversible inactivation and competitive inhibition of the decarboxylases and the dehydrogenase. These analogs include α-difluoromethyldiaminopimelate, N-hydroxydiaminopimelate, all stereochemically pure forms of sulfur-containing analogs, lanthionine, lanthionine sulfoxide, and lanthionine sulfone. Surprisingly, the difluoromethyl compound is neither a suicide substrate nor a good competitive inhibitor, in contrast to the effect of such derivatives on other decarboxylases. The remaining analogs, meso-lanthionine and its sulfoxide, are modest competitive substrates. However, the N-hydroxy derivative is a potent competitive inhibitor of the decarboxylase and binds about ten times more strongly than does the natural substrate. Preliminary microbiological tests for antibiotic activity with the N-hydroxydiaminopimelate will be initiated soon.

Other work by Dr. Vederas on the preparation of diaminopimelate analogs has led to the development of a general stereospecific synthesis of α-hydrazino acids. These compounds are potent specific inhibitors of a variety of enzymes and can also be converted to the corresponding α-amino acids by reduction with Raney nickel. The α-hydrazino acids are formed by coupling the parent acids to readily available chiral oxazolidones, followed by oxidation of the corresponding enolates with alkyl azodicarboxylates and deprotection. The method will be used to synthesize diaminopimelate analogs as possible inhibitors of the dehydrogenase and decarboxylases of the diaminopimelate pathway and hopefully would be useful broad spectrum antibacterials with low mammalian toxicity.

Models of Enzyme Reactions

"Host Molecules that Complex and Catalyze"
R01 GM 12640-21 (Cram, D.), University of California, Los Angeles

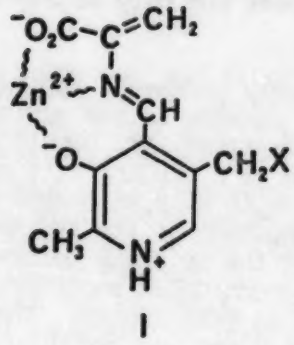
A powerful approach for learning about the physical-chemical basis of health is the design of organic chemical models for chemical reactions catalyzed by enzymes. The transacylases are important in the building and degradation of proteins and fats. The active sites of transacylase enzymes isolated from a variety of organisms

appear to contain concave complexing sites on whose rims are gathered a hydroxyl, an imidazole, and a carboxyl, which act cooperatively to catalyze acyl transfer reactions. The support structures that provide organization to the transacylase active sites are peptide in character but vary widely in their molecular weights and amino acid sequences.

Based on the hypothesis that entirely different support structures can be used to synthesize transacylase catalysts as long as the active sites are organized similarly to those of the enzymes themselves, Dr. Donald Cram has designed a potential artificial transacylase consisting of a binding site and the three functional groups of the natural transacylases in an arrangement resembling that of the natural enzyme. An incremental approach to its synthesis was devised to test the performance of each portion of the molecule before the four parts were fully assembled. The binding site was constructed successfully and found to collect and orient the reacting molecules as anticipated. The hydroxyl group that accepts the acyl group was next put in place and was found to perform its assigned task as acyl acceptor. Most recently, in a 30-step synthesis, the imidazole group was located properly in the desired synthetic catalyst, with dramatic results. The compound acted as an acyl acceptor in the absence of an external base. The observed acetylation reaction rate was 100,000 times faster than that measured when the various catalytic parts were unassembled. Although the final carboxyl group has not yet been introduced into the compound, the acylated catalyst undergoes slow hydrolysis to provide catalytic turnover. Dr. Cram hopes that addition of this carboxyl group will accelerate the hydrolytic rate.

"A Mimic of Tryptophan Synthetase"
R01 GM 18754-24 (Breslow, R.), Columbia University

Dr. Ronald Breslow and his coworkers in the Department of Chemistry at Columbia University have synthesized a molecule that imitates the ability of tryptophan synthetase to bind two components and assemble them, forming a new carbon-carbon bond. The synthetic catalyst is a cyclodextrin molecule carrying a pyridoxal unit (see structure II) and is related to a cyclodextrin pyridoxamine species synthesized in the Breslow laboratories a few years ago. In tryptophan synthetase, a pyridoxal cofactor binds a serine residue and promotes the formation of a dehydrated derivative that reacts with indole held in a binding pocket to form tryptophan. In the artificial enzyme prepared at Columbia, the pyridoxal unit binds an amino acid which forms the aminoacrylic acid derivative compound 1, while the cyclodextrin unit functions as a binding pocket to hold the indole in position to react with the aminoacrylic acid derivative. The system catalyzes a reaction starting with serine but is more effective when the starting material is chloroalanine. The latter compound generates the aminoacrylic acid intermediate 1 more readily with fewer side reactions. Evidence for this mechanism is obtained from the fact that the cyclodextrin-pyridoxal catalyst gives higher yields than does a simple pyridoxal derivative, and also from the fact that the product tryptophan has some optical activity. This suggests that the product is formed inside the chiral cyclodextrin unit. Dr. Breslow is attempting to improve the catalyst by adding an additional basic catalytic group and eliminating some of the flexibility in the system.



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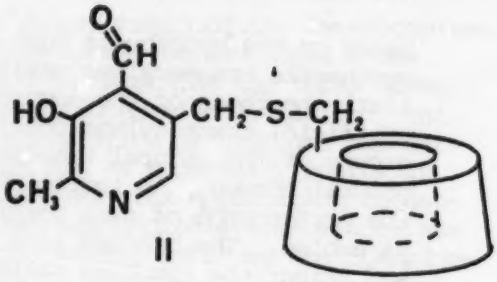

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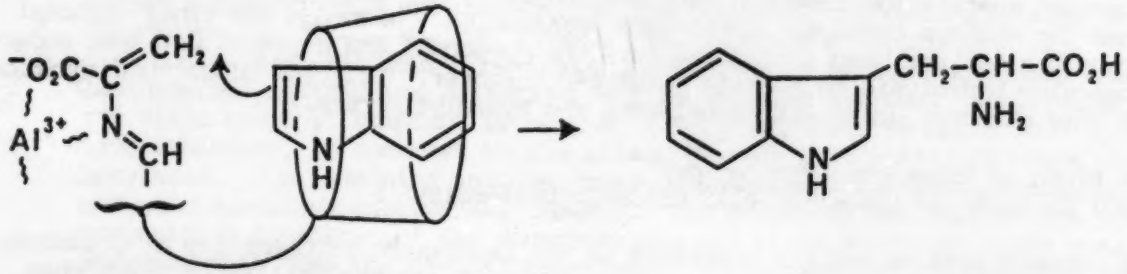


"Synthesis of Vitamin B_{12} Models"
R01 GM 19906-09 (Dowd, P.), University of Pittsburgh

Vitamin B_{12} is an obligatory cofactor in at least ten enzyme-catalyzed rearrangement reactions. Of these, three are carbon-skeleton rearrangements:

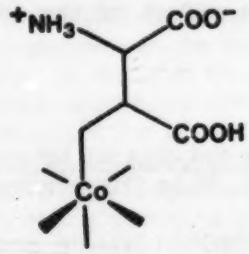
β-methylaspartate ⇄ glutamate
methylmalonyl-SCoA ⇄ succinyl-SCoA.
methylitaconate ⇄ α-methyleneglutarate

Unlike most enzyme-catalyzed reactions, there have been no known analogous rearrangements in organic chemistry; hence, there exists a great deal of uncertainty about the mechanism of the enzyme-catalyzed rearrangement reactions. As the methylmalonyl-SCoA reaction is a major one in human nutrition and metabolism, it is important to understand it better.

Dr. Paul Dowd and his research group are developing models and reactive intermediates for reactions involving vitamin B_{12} as a cofactor. One of their first major findings was the fact that the adduct of methylitaconate attached to the cobalt atom of vitamin B_{12} decomposes spontaneously in aqueous solution, in the dark, and at physiological pH, yielding rearranged α-methyleneglutaric acid. These conditions are ideal for a biological model and provide the first experimental

support for the notion that the carbon-cobalt substrate bond might be the key element of the rearrangement.

Recently, Dr. Dowd constructed an adduct between β-methylaspartic acid and vitamin B_{12}. This is the first adduct of a completely unprotected substrate



attached to cobalt and will help to test the hypothesis that the carbon-cobalt bond is a central intermediate in carbon-skeleton rearrangement reactions. In the course of his research, Dr. Dowd found β-methyleneaspartic acid, a precursor to the β-methylaspartic acid--vitamin B_{12} adduct, to be a highly specific inhibitor of glutamate-aspartate transaminase.

Dr. Dowd has extended his research to include a model for the rearrangement reaction between methylmalonate and succinate and has been exploring the reactivity of the B_{12}-methylaspartic acid adduct to learn whether its rearrangement will also occur outside the enzymic milieu. His model for the attachment of substrate to cobalt also is being used by others for further study of the methylmalonate reaction, as well as for the coenzyme B_{12}-dependent ethylene glycol-to-acetaldehyde rearrangement.

"Models for Biological Redox Processes"
R01 GM 32296-02 (Meyer, T.), University of North Carolina at Chapel Hill

Dr. Thomas Meyer has found that mechanisms for sophisticated biological reactions can be copied with well defined and relatively simple metal coordination complexes. A range of simple complexes may achieve to some extent the catalytic chemistry of a biological enzyme, even though the complexes involved show limited, if any, obvious structural similarity to enzymes.

One such complex is the dimeric ruthenium species $(2,2'\text{bipyridine})_2(H_2O)Ru^{III}ORu^{III}(H_2O)(2,2'\text{bipyridine})_2$. In its oxidized form, the complex is capable of oxidizing water to give oxygen, making it an analog of the terminal site in photosystem II, which "splits water" in photosynthesis. Mechanistic studies suggest that part of the oxygen produced is from water molecules initially bound to the two ruthenium atoms, and part arises from solvent molecules. Understanding the process by which the complex catalyzes formation of the oxygen-oxygen bond should shed considerable light on the mechanism of action of photosystem II. The reduction of nitrite to ammonia is a complex six-electron/seven-proton reduction carried out by the enzyme nitrite reductase in a variety of living cells. Dr. Meyer has found that a water soluble iron porphyrin, which structurally is very similar to the active site of nitrite reductase, can catalyze this reaction via a nitrosyl intermediate. In fact, Dr. Meyer found that a simple coordination complex of iron and the industrially important ligand EDTA (ethylene

diamine tetraacetic acid) also is effective in generating from nitrite a nitrosyl intermediate in which the NO ligand can be reduced to ammonia. Dr. Meyer is studying the mechanism by which these catalysts operate and is comparing his results with previous mechanistic studies on the stoichiometric reduction of coordinated nitrite to ammonia by polypyridine complexes of ruthenium and osmium. His observations suggest that the reductive chemistry occurs on the metal-nitrite/nitrosyl fragment and that the effect of the additional ligands is secondary.

Anesthesia Mechanisms

"Anesthesia-Analgesia of Awake Cat Spinal Neurons"
R01 GM 29065-05 (Collins, J.), Yale University

Dr. J. Collins is seeking to explain how an anesthetic produces anesthesia. Toward this goal, he has developed a neurophysiological recording technique for measuring extracellular single unit activity from neurons in the dorsal horn of the spinal cord. Dr. Collins has used this technique to measure the effects of anesthetic agents on the spontaneous activity in low threshold neurons, i.e., those neurons responsible for signalling low intensity tactile information. In normal, intact animals, these neurons have little or no spontaneous activity, but high levels of activity are seen in decerebrate, spinal cord transsected, or anesthesized animals. Specifically, in intact, drug-free animals, no low threshold neurons had spontaneous firing rates greater than two impulses per second, and many neurons had spontaneous firing rates less than 0.5 impulses per second. In contrast, in pentobarbital anesthetized animals (20mg. per kg. I.V.), approximately 1/3 of the neuronal population that have been recorded have spontaneous firing rates greater than two impulses per second, with some neurons demonstrating rates of 10-15 impulses per second.

The absence of spontaneous activity in control animals suggests the existence of descending modulatory influences that inhibit spontaneous activity. The presence of spontaneous activity in low threshold neurons in anesthetized animals, and the knowledge that inhibitory systems may be more susceptible to anesthetic actions than excitatory systems, suggests that the spontaneous activity seen in anesthetized animals may be due to anesthetic inhibition of descending inhibitory systems. Release of spontaneous activity at the spinal level by barbiturates may provide a partial explanation for changes in electrical activity that are seen in supra-spinal sites during clinical anesthesia.

The signalling capability of the central nervous system is assumed to be based upon the presence of a signal embedded in noise (spontaneous activity). Thus, the increased spontaneous activity that occurs with barbiturate anesthesia may produce a noise level which interferes with normal signal processing, thereby disrupting the ability of the central nervous system to communicate with the periphery.

"Anesthetic Interaction with Model Proteins"
R01 GM 26950-06 (Ueda, I.), University of Utah

It has been postulated that inhalation anesthetics weaken water-macromolecule associations, either by directly residing at the interface or by indirectly decreasing the interfacial density of hydrophilic moieties of macromolecules.

Dr. Issaku Ueda's research has focused on changes in the interfacial properties of proteins and model macromolecules that are produced by inhalation anesthetics. Previously, indirect evidence obtained by high precision solution densimetry and thermodynamic arguments indicated that inhalation anesthetics cause the release of water molecules from the surface of bovine serum albumin dissolved in water. Recently, Dr. Ueda obtained more direct evidence, by proton nuclear magnetic resonance spectroscopy, that anesthetics do cause the release of tightly bound water. The dynamic state of water molecules can be visualized in NMR spectra by the width of the proton signal that is related to spin-spin relaxation time to the first approximation. When water molecules are freely moving, their proton signal is steeper. Because the large quantity of bulk water in aqueous solutions obscures identification of bound water, Dr. Ueda performed his study in a water-in-oil reversed micelle system, prepared by dispersing a small amount of water in decane doped with an anionic surfactant (glycerol-alpha-monooleate). An increase of the half-height line-width of the water signal was observable at enflurane concentrations as low as $8 \cdot 10^{-5}$ M. This result is consistent with an anesthetic-induced release of electrostricted water, which is water attracted to the protein surface and held in a crystalline structure.

"Axonal Study for Improved Selective Nerve Anesthesia"
R01 GM 31710-03 (Fink, B.), University of Washington

Studies of the effects and mode of action of local anesthetics have been based on the premise that decreases in electrical waveform amplitudes were proportional to the number of fibers blocked in a given fiber group. Dr. B. Raymond Fink's research with individual fibers has shown this to be incorrect: low concentrations of anesthetic that block only a very small percentage of individual fibers make a much larger change than expected in the amplitude of the relevant compound waveform. Furthermore, contrary to inferences from studies of compound potentials, Dr. Fink's research has shown that neither the margin of safety of conduction nor the anesthetic concentration required to block myelinated fibers of various sizes (which serve such diverse functions as pain, touch and motor activity) are related directly to the conduction velocity or size of the fiber. Thus, the well-attested clinical differential action of anesthetics on functions mediated by myelinated axons is not explicable by size-related differential sensitivity to block, as previously supposed, but must involve something else. Understanding of that 'something else' will be necessary for further progress in differential control of pain with drugs acting on nerves.

Dr. Fink also is studying the effects of local anesthetics that are used in childbirth, where the objective is to relieve pain without impairing the action of the expulsive muscles. For reasons that have been unclear, this objective is partially realized with epidural anesthesia, in which the drug is deposited within the vertebral canal but outside the covering of the spinal cord. As part of a project to determine why epidural anesthesia has a greater effect on pain fibers than on motor fibers, Dr. Fink used autopsy tissue to measure the length of spinal nerve root traversing the region where the epidural anesthetic is deposited. This zone extends from the sac covering the spinal cord to the gaps between the vertebrae through which the nerves leave the spinal canal. Nerve roots in this zone range from 5 to 10 millimeters long. The minimum length of

nerve fiber that needs to be in contact with local anesthetic in order to obtain block is known to increase with the size of the fiber and may be as much as 8 mm. Futhermore, motor nerve fibers supplying muscles generally are larger than those serving pain. Thus, the epidural differential block may arise, at least in part, because the site of administration provides drug access to a length of nerve root that is insufficient to block conduction in the motor fibers but is more than sufficient to block conduction in the pain fibers.

The insights obtained in this project have resulted in a systematic classification of clinically observed differential blocks and their mechanisms, providing a rational approach to epidural techniques for using local anesthetics and opioids in managing chronic, acute, and subacute abdominal pain.

"Local Anesthesia of Peripheral and Central Nerve Pathways"
R01 GM 35647-01 (Strichartz, G.), Brigham and Women's Hospital, Boston

Dr. Gary Strichartz is seeking to define conditions and factors that affect the potency of local anesthetics in blocking peripheral nerve fibers. In studies on nerve impulse amplitudes and impulse thresholds, the potency of lidocaine, an often used local anesthetic, was shown to depend on solution acidity (pH) and on the buffers used. In particular, potency increased when the pH was increased with organic buffers (HEPES) but fell when the pH was increased with CO_2-bicarbonate buffer. This result demonstrates the importance of buffer choice in studying anesthetic mechanisms and reveals a possible pharmacological activity of CO_2-bicarbonate.

Dr. Strichartz also found that cooling the nerves from the normal temperature of 30° to 20-15° dramatically potentiated anesthetic action. Phasic blocking activity was particularly enhanced. The additional block disappeared rapidly upon rewarming. Such local cooling could be a very useful addition to current local anesthetic techniques.

In research to determine the dependence of anesthetic structure on the rate and potency of isolated nerve block, Dr. Strichartz found that anesthetic molecules containing ester linkages tend to be more potent than those with amide linkages. With respect to the tertiary amine region of these drugs, molecules with greater hydrophobicity, due to the type of alkyl substituents on this nucleus, were more potent for blocking single impulses but reached a maximum for phasic block at intermediate hydrophobicities. These studies are likely to aid the future design of local anesthetics intended for various specific procedures.

Anesthesia: Interactions and Metabolic Effects

"Anesthetic Reactions in Surgery"
R01 GM 31382-02 (Louis, C.), University of Minnesota

Malignant hyperthermia is thought to be triggered by an uncontrollable release of intracellular calcium in muscle, resulting in a massive increase in metabolic rate and muscle contracture. A defect in the ability of muscle surface membranes to control calcium movement could contribute to the onset of malignant hyperthermia. Dr. Charles Louis is employing sealed vesicles from the muscle membranes of pigs that are susceptible to malignant hyperthermia and is determining whether

their calcium permeability is abnormal. These vesicles contain an ATP-dependent calcium pump whose affinity for calcium appears capable of lowering sarcoplasmic calcium to the levels present in relaxed muscle. Furthermore, the activity of this pump was found to be regulated by both the calcium receptor calmodulin and the cyclic nucleotide receptor, cyclic AMP-dependent protein kinase. Thus, transport of calcium across muscle surface membranes appears to be sensitive to the circulating levels of hormones. In addition to the ATP-dependent calcium transport process, muscle surface membranes also contain a sodium-calcium exchange system that could be one of the multiple mechanisms by which muscle surface membranes regulate intracellular calcium levels. As the sodium-calcium exchange process seems to be regulated by ATP, this could have implications in malignant hyperthermia where muscle ATP levels are depressed.

Dr. Louis compared the mechanism regulating calcium release from the sarcoplasmic reticulum of malignant hyperthermia muscle with that of control muscle. After passively loading the sarcoplasmic reticulum vesicles with calcium, calcium release can be induced from the vesicles by a sudden elevation in extracellular calcium. This release of calcium is enhanced by a number of agents, including caffeine and ATP, and is inhibited by others, including $MgCl_2$ and ruthenium red. The calcium concentration at which this release is half-maximal is in the micromolar range, which is in the range of calcium concentrations observed during muscle contraction in vivo. For sarcoplasmic reticulum from animals exhibiting malignant hyperthermia, Dr. Louis found that calcium release is triggered at a lower external calcium concentration, and the amount of calcium released is greater, than that from sarcoplasmic reticulum of control animals. Thus, there seems to be a defect in the control of the calcium release process in pigs susceptible to malignant hyperthermia. Whether this defect is in the sarcoplasmic reticulum or in the transverse tubules communicating with the sarcoplasmic reticulum is under study.

This research should provide a better understanding of muscle function in malignant hyperthermia which, in turn, should lead to improved procedures for its diagnosis and treatment.

"Catecholamine-Halothane Interactions During Anesthesia"
R23 GM 30232-03 (Maze, M.), Stanford University

Halothane sensitizes the heart to the arrhythmogenic effects of epinephrine. The concurrent administration of a methylxanthine compound, such as aminophylline, for its bronchodilatory effect during halothane anesthesia may enhance this arrhythmogenic interaction because of its sympathomimetic effects. Previous animal studies of this interaction have limited clinical validity because aminophylline was administered as a single bolus dose, causing continuously varying methylxanthine levels.

Dr. Mervyn Maze has designed a study to determine the effect of aminophylline on halothane-epinephrine arrhythmias under conditions of a standardized, more clinically relevant mode of aminophylline administration. In these studies, no ventricular arrhythmias were observed without the administration of exogenous epinephrine, regardless of plasma methylxanthine concentration. However, aminophylline enhanced the sensitization by halothane to the arrhythmogenic effects of epinephrine, even at therapeutic levels. Because arrhythmias occurred at significantly lower plasma levels of epinephrine when aminophylline was present,

this arrhythmia-sensitizing effect probably is mediated by a pharmacodynamic and not a pharmacokinetic alteration.

Halothane is frequently selected for an anesthetic in patients with obstructive pulmonary disease because of its potent bronchodilatory action. If these data are extrapolated to humans, the concurrent perioperative administration of aminophylline increases the risk for developing ventricular arrhythmias. Intraoperative administration of aminophylline to treat acute bronchospasm in the halothane anesthetized patient may constitute an even greater risk, since endogenous catecholamine levels may be elevated due to hypoxia or hypercarbia.

"Paralysis—Its Antagonism During Surgery and Anesthesia"
R01 GM 26403-06 (Miller, R.), University of California, San Francisco
"Pharmacokinetics, Muscle Relaxants and Antagonists"
R01 GM 26745-06 (Matteo, R.), Columbia University

Patients who receive general anesthesia for surgery also are given neuromuscular blocking drugs such as pancuronium, succinylcholine, vecuronium or atracurium, to facilitate anesthesia and surgery. These drugs induce paralysis, which allows complete relaxation with light levels of anesthetic drugs. The most important complication of the neuromuscular blocking drugs is prolonged paralysis and inadequate ventilation in the postoperative period. This complication has prompted research directed toward determining how these drugs are eliminated from the body and what pathologic conditions influence their rate of excretion.

Dr. Ronald Miller has found that pancuronium is eliminated primarily through the kidney; therefore, its effect is markedly prolonged in patients undergoing renal transplantation. Conversely, only 10 to 20 percent of an injected dose of vecuronium is eliminated through the kidney, while atracurium is metabolized and is not dependent on the kidney or liver for its elimination.

The neuromuscular blockade (paralysis) induced by these muscle relaxants can be antagonized by inhibitors of acetylcholinesterase, such as edrophonium, neostigmine and pyridostigmine. Dr. Miller found edrophonium to have a very rapid onset (1 to 2 minutes) in comparison to neostigmine, which has an onset of 6 to 7 minutes, and pyridostigmine, which has an onset of greater than 12 minutes. Edrophonium requires about half the amount of atropine to prevent bradycardia as compared to that required by neostigmine or pyridostigmine. However, when the neuromuscular blockade is greater than 90 percent, edrophonium is an ineffective antagonist. In these situations, neostigmine or pyridostigmine should be used.

Dr. Richard Matteo and others found previously that the effect of most muscle relaxants is prolonged in elderly patients. This is due primarily to decreased volumes of distribution and decreased urinary excretion of the relaxants in patients over age 70 years. Dr. Matteo recently found that the action of pyridostigmine and edrophonium are also significantly prolonged in elderly patients. This latter effect seems to be related to a decreased volume of distribution and a decreased plasma clearance for the drugs. Studies such as these by Drs. Miller and Matteo should help to decrease the incidence of prolonged neuromuscular blockade and inadequate ventillation during the postoperative period and also should lead to a better understanding of changes in drug actions and metabolism that occur with advancing years.

"Dialysable Component(s) of Uremic Blood Potentiate the Hypnotic Effect of Phenobarbital"
P50 GM 20852-12 (Levy, G.), State University of New York at Buffalo

Studies in Dr. Gerhard Levy's laboratory have shown that rats with renal dysfunction are significantly more sensitive than normal animals to the hypnotic effect of phenobarbital. These observations are consistent with earlier clinical observations by others of decreased thiopental dose requirements for induction and maintenance of general anesthesia in azotemic patients. To explore the mechanism of the increased sensitivity of the central nervous system to the hypnotic effect of barbiturates during renal dysfunction, Dr. Levy administered intravenously a concentrate of serum dialysate from either normal rats or from rats made uremic by the earlier administration of uranyl nitrate. The rats also received a slow intravenous infusion of phenobarbital until they lost their righting reflex. Rats infused with serum dialysate from uremic rats lost their righting reflex at significantly lower phenobarbital concentrations in their serum, brain and cerebrospinal fluid than occurred for rats given serum dialysate from normal rats. Thus, the serum of uremic rats contains appreciable concentrations of one or more dialysable components capable of potentiating or contributing to the hypnotic activity of phenobarbital. Experiments designed to reveal the nature of the substance(s) so far have shown that it is not urea. These results raise the possibility that the response of patients with renal failure to a barbiturate may vary, depending on the time elapsed since their last dialysis treatment.

"Anesthetic Effects on Viral Replication or Pathogenesis"
K04 GM 00477-05 (Knight, P.), University of Michigan, Ann Arbor

The replication of measles, herpes, vesicular stomatitis, Newcastle disease, and SV_{40} viruses are all known to be inhibited to some degree by exposure to halothane. Synthesis of poliovirus is not affected. Dr. Paul Knight currently is analyzing the effects of halothane on the synthesis of measles virus protein, using polyacrylamide gel electrophoresis and immunofluorescence staining techniques. Two glycosylated measles virus proteins that are intimately associated with the cellular membrane are not readily detectable in the presence of 2.0 percent halothane. This inhibition is at least partially reversed by hyperbaric conditions. The synthesis of several nonmembrane associated measles virus proteins are inhibited to varying degrees, and the inhibition of these virus proteins is not reversed by hyperbaric conditions.

Halothane also inhibits herpes virus replication and protein synthesis. Hyperbaric conditions do not reverse the inhibition of herpes virus replication produced by halothane, but instead enhance the inhibition. These results suggest that halothane inhibits herpes virus replication by a mechanism different from that which occurs for measles virus.

A prospective cohort study involving pediatric patients is under way to study the effect of anesthesia in patients with viral infections. Analysis of data to date has revealed no increase in morbidity in patients anesthetized in the presence of a viral upper respiratory tract infection.

These studies may provide clinically useful data in determining management tactics for anesthesia with patients harboring a viral infection. Furthermore,

analysis of the inhibition of virus protein synthesis and particle assembly by volatile anesthetics in the presence and absence of hyperbaric conditions provides insight into the molecular mechanism of anesthetic action.

In conjunction with the virus protein studies, Dr. Knight has also been studying the effects of anesthetics on cellular macromolecular synthesis. Short term exposure of cell cultures to halothane (less than 6 hours) did not inhibit cellular protein synthesis, but longer term exposure caused a dose-related inhibition.

"Control of Intracranial Pressure During Anesthesia"
R01 GM 33006-02 (Artru, A.), University of Washington

For patients with head trauma, hyperventilation is used to decrease elevated intracranial pressure, thereby minimizing the likelihood of brain damage. Patients with head trauma who are being treated by hyperventilation often require anesthesia for surgery to repair life-threatening effects of trauma. Without anesthesia, the intracranial pressure-lowering effect of hyperventilation lessens over a period of hours and may be lost with time, so that patients again are at risk for brain damage. Dr. Alan Artru is determining whether commonly used anesthetics alter the changes in intracranial pressure that are induced by hyperventilation, using procedures that permit the simultaneous determination of intracranial pressure, cerebral blood volume, and cerebrospinal fluid volume.

With no potent anesthetic present and intracranial pressure initially normal, hyperventilation maintains intracranial pressure at low values over four hours. With fentanyl anesthesia and intracranial pressure initially elevated, hyperventilation causes a 50 percent reduction in intracranial pressure, which is sustained for 2.5 hours, with intracranial pressure increasing to a 30 percent reduction by four hours. No expansion of cerebral blood volume over four hours was seen, but a slight expansion of cerebrospinal fluid volume was detectable after 2.5 hours. With halothane anesthesia and the intracranial pressure initially elevated, hyperventilation caused only a slight reduction of intracranial pressure, and by four hours the intracranial pressure was elevated by about 30 percent. Both the cerebral blood volume and the cerebrospinal fluid volume expanded during the four hours of hyperventilation. In contrast, with no potent anesthetic present but with the intracranial pressure initially elevated by an intracranial mass, hyperventilation results in a 30 percent reduction in intracranial pressure that is sustained over four hours. The cerebral blood volume expanded by about 1 ml and the cerebrospinal fluid volume contracted by the same amount over four hours.

Thus, fentanyl slows the recurrence of elevated intracranial pressure during hyperventilation via cerebrovasoconstriction. In contrast, halothane augments the recurrence of elevated intracranial pressure during hyperventilation. The cerebral blood volume expansion in the presence of halothane was similar to that seen in the absence of a potent anesthetic, and the cerebrospinal fluid volume also was increased, indicating impaired reabsorption.

This work should demonstrate which anesthetic(s) are safest for patients being hyperventilated to control intracranial pressure and also should provide information as to the mechanisms that are responsible for the recurrence of elevated intracranial pressure.

"Alterations in Neuromuscular Function Following Burns"
R23 GM 31569-02 (Martyn, J.), Massachusetts General Hospital

Burned patients have altered responses to neuromuscular blocking drugs. They can develop hyperkalemia and cardiac arrest with the administration of depolarizing relaxants such as succinylcholine, and they are resistant to the effects of non-depolarizing relaxants such as d-tubocurarine.

Dr. Jeevendra Martyn and his colleagues have examined the effects of burns on the dose response curves of adults and children. Resistance to non-depolarizing relaxants is usually not seen in patients sustaining burns of less than 10 percent body surface area; however, patients with greater than 40 percent body burn may need doses that are 2.5-3 fold higher. Thus, initial dosages for d-tubocurarine, metocurine and pancuronium would be 1.8, 0.8 and 0.13 mg/kg, respectively, in burned patients compared to 0.51. 0.3 and 0.05 mg/kg, respectively, in non-burned subjects. Patients with intermediate size burns (15-40 percent) require intermediate doses. These increased doses are required despite the concomitant administration of aminoglycoside antibiotics, which are known to potentiate the neuromuscular effects of muscle relaxants. Dr. Martyn and his colleagues demonstrated by equilibrium dialysis techniques that many drugs, including muscle relaxants, have increased binding (decreased free fraction) to plasma protein despite burn-induced decreases in total protein and albumin concentrations. The increased binding appears to be due to the increase in $alpha_1$-acid glycoprotein that occurs after burns.

Substitutes for succinylcholine are needed that produce rapid onset neuromuscular paralysis in the burned patient for emergency situations (full stomach, laryngospasm). High doses of non-depolarizers can cause rapid onset of paralysis in normal subjects. However, burned patients require even higher doses because of hyposensitivity, and there are unacceptable cardiovascular side effects. Studies in normal adults have demonstrated significant cardiovascular stability and rapid onset of paralysis when pancuronium and metocurine are administered in combination. When three times the effective doses of pancuronium and metocurine were administered to burned patients, the mean onset time was 1.4 minutes, but prolonged paralysis resulted.

The recently introduced atracurium and vecuronium offer some advantages over the older relaxants, as they have a shorter duration of action and minimal cardiovascular effects. Further studies are needed to determine whether manipulation of dosage or combination with other relaxants will speed onset of their effects in healthy or in burned patients.

Anesthesia: Cardiovascular Effects

"Mechanisms of Contractile Depression by Volatile Anesthetics"
R23 GM 31144-03 (Lynch, C.), University of Virginia

In order to determine the mechanism by which halothane, enflurane, and isoflurane depress myocardial contractility, Dr. Carl Lynch examined the effects of these volatile anesthetics on slow action potentials, which are mediated by calcium ion influx through the slow channel. Since calcium ions, which activate contraction, appear to arise from different sources at different rates of stimulation, he

measured the pattern and magnitude of contraction at different stimulation frequencies for both normal and slow action potentials. He also compared the effects of anesthetics with drugs of the following types: 1) calcium entry blockers such as nifedipine, verapamil, and diltiazem; 2) drugs that inhibit calcium release from its internal store (the sarcoplasmic reticulum), such as the local anesthetics tetracaine and procaine, and the skeletal muscle relaxant, dantrolene sodium.

Dr. Lynch found that the slow action potential rate of depolarization, which approximates calcium ion influx through the slow channel, is depressed less by isoflurane than by halothane or enflurane. At equivalent anesthetic concentrations, both halothane and enflurane were also more depressant than isoflurane to the early-peaking contraction at higher frequencies (2-3 per second). Calcium entry blockers showed similar effects to halothane and enflurane in depressing peak tension and the initial rate of tension development. In contrast, tetracaine showed little or no depression of early-peaking tension at higher frequencies and no depression of the slow action potential rate of depolarization, a measure of calcium ion influx. Tetracaine markedly depressed the late peaking tension and the rate of late tension development observed at frequencies of one per second or less. Isoflurane, enflurane, and dantrolene showed similar depression of the late peak tension, which is attributed to calcium release by the sarcoplasmic reticulum. Both dantrolene and isoflurane appear to have modest calcium entry blocking activity and also cause a 10-25 percent depression of the early peaking tension at high frequency.

Thus, isoflurane differs dramatically from halothane by depressing contractility at low (non-physiologic) frequencies by depressing calcium release from the sarcoplasmic reticulum. While enflurane has similar depressant properties, it is like halothane in its ability to depress contractility more than isoflurane at higher (physiologic) frequencies, and in its more profound depression of slow action potential rates of depolarization (a measure of calcium entry) and contractions at physiologic frequencies. Halothane and enflurane appear to block calcium entry. Experiments combining calcium entry blockers with halothane or isoflurane show the latter combination to be less depressant, as predicted by the drugs' individual effects on calcium ion fluxes.

"Anesthetic Induced Cardiac Depression"
RO1 GM 29527-03 (Rusy, B.), University of Wisconsin, Madison

General anesthetics depress the ability of the heart to contract. For this reason, Dr. Ben Rusy is examining the effect of isoflurane on rested-state, potentiated-state, and steady state muscle contractions. Isoflurane markedly depresses rested-state contractions, which depend primarily on calcium ion derived via transsarcolemmal influx. Potentiated-state contractions, which are dependent on calcium ion derived via internal release, are affected significantly less. These results suggest that isoflurane's negative inotropic action is mediated mainly by depressing the transsarcolemmal influx of calcium ion but that the anesthetic probably has only a small effect on internally released calcium ion. Comparison of isoflurane's effect with that of nickel ion, a specific inhibitor of calcium ion influx, and of ryanodine, a specific inhibitor of calcium ion release from the sarcoplasmic reticulum, supports this suggestion. Preliminary studies suggest that isoflurane's negative inotropic effect can be effectively reversed by isoproterenol, which increases the transsarcolemmal influx of calcium ion.

Dr. Rusy used a similar protocol to evaluate the effects of etomidate, an intravenous anesthetic induction agent. This drug has only a small negative inotropic effect that appears to be mediated primarily by inhibiting transsarcolemmal calcium ion influx. The depressant effect of etomidate is readily reversed by increasing the concentration of extracellular calcium ion. Knowledge about anesthetic effects on calcium ion sources and movement in cardiac tissue, such as that obtained in this study, should facilitate the development of successful pharmacological approaches for preventing or reversing anesthetic-induced myocardial depression.

"Organ Protection: Role of Anesthetics"
R01 GM 33137-03 (Tinker, J.), University of Iowa

Dr. John Tinker and his collaborators have developed techniques for studying coronary artery flow velocity, using a 20 MHz doppler ultrasonic flow velocity probe. They found large and important differences in coronary artery flow velocity produced by halothane and isoflurane, with the latter agent much better able to preserve vascular reactivity in the coronary circulation than the former. They conclude that halothane has a specific direct vascular toxic effect. This finding could be a useful criterion in choosing anesthesia for cardiac patients.

This group also has been studying toxic therapeutic ratios, i.e., the dose of anesthesia which produces obvious toxicity such as cardiac arrest or fibrillation, divided by the dose at which movement is lost in response to skin incision. Isoflurane was found to have approximately double the toxic therapeutic ratio of halothane, which would indicate that isoflurane is a safer anesthetic than halothane. Measurements performed in the presence or absence of surgery and in the presence or absence of a critical coronary stenosis gave the same results.

"Hypoxic Pulmonary Vasoconstriction (HPV) and General Anesthesia"
R01 GM 29628-04 (Marshall, B.), University of Pennsylvania

Inhalational anesthetics consistently inhibit hypoxic pulmonary vasoconstriction of *in vitro* lung preparations, but the results have been contradictory *in vivo*. Dr. Bryan Marshall has combined information on quantitative variables affecting hypoxic pulmonary vasoconstriction with a model of the pulmonary circulation based on anatomic characteristics of the pulmonary vessels in the lung and is using this combined model to explore by computer the variables known to interact with hypoxic pulmonary vasoconstriction during general anesthesia. The results of the computer simulation approximate those reported *in vivo* and, for the first time, show why the fundamental inhibition of hypoxic pulmonary vasoconstriction by inhalational anesthetics may not be detectable *in vivo*. The reason is that direct inhibition of hypoxic pulmonary vasoconstriction in the presence of isoflurane, halothane or enflurane is offset by simultaneous changes of cardiac output, mixed venous oxygen tension and pulmonary venous pressure that tend to promote hypoxic pulmonary vasoconstriction. This computer simulation is helping to provide a more complete understanding of the effects of anesthetic gases and is proving valuable both in designing definitive new studies and in applying research results to patients.

"Anesthetic as Potentiator of Mediator Release in Lungs"
R01 GM 32262-02 (Gurtner, G.), Johns Hopkins University

Dr. Gail Gurtner's research is designed to aid understanding of the augmentation of the vasoconstriction caused by 300 μM t-butyl hydroperoxide at different Minimum Alveolar Concentration (MAC) levels of anesthetic agents having widely differing anesthetic potencies. In studies with halothane, which has a MAC of .76 percent, cyclopropane, which has a MAC of 9.2 percent, and nitrous oxide, which has a MAC of 105 percent, she found that each agent augmented the pressor response similarly in proportion to MAC. Thus, augmentation of thromboxane production, which mediates the pressor response, as well as anesthetic potency, is a membrane effect.

Dr. Gurtner also investigated the site of potentiation of thromboxane production within the cyclooxygenase pathway. She measured the effect of quinacrine (mepacrine), a known inhibitor of phospholipase A2 activity, and indomethacin on oxidant induced pulmonary hypertension, with and without anesthetic agent. She found that this drug completely inhibited the pressor response and the production of thromboxane, supporting the hypothesis that the site of anesthetic potentiation of the pressor response is the first enzymatic reaction of the arachidonic acid cascade.

Dr. Gurtner found that thromboxane-mediated pulmonary vasoconstriction is calcium-dependent in that it can be completely inhibited by calcium channel blockers or by perfusing the lung with calcium-free perfusate. Thromboxane production itself is not affected by these interventions. Thromboxane-mediated pulmonary vasoconstriction is also blocked by the calmodulin inhibitors chlorpromazine and trifluperazine, indicating that calmodulin is involved in the contractile process.

In studying the role of leukotrienes in lung injury, Dr. Gurtner found that administration of leukotriene D4 increases lung permeability, as well as pulmonary artery and airway pressure. All effects of leukotriene D4 can be blocked by FPL-55712, a known leukotriene receptor blocker. The pressor and airway effects probably are caused by secondary stimulation of cyclooxygenase activity, since they can be blocked by indomethacin. However, permeability changes are unaffected by indomethacin administration and probably represent a direct effect.
Dr. Gurtner's results also show that inhibitors of the lipoxygenase pathway protect against oxidant lung injury during challenge with t-butyl hydroperoxide. These experiments support the hypothesis that lipoxygenase mediators are important in the pathophysiology of lung injury.

Anesthetics and Development

"Anesthetic-Induced Alterations of Early Development"
R01 GM 31723-02 (Hinkley, R.), University of Miami

Dr. Robert Hinkley is using sea urchin gametes as a model system to study the effects of volatile anesthetics on development. He has found that halothane induces the acrosome reaction in sperm, inhibits sperm-egg binding, inhibits the growth of the sperm aster, and may delay or prevent syngamy (fusion of the male and female pronuclei). When present at the time of fertilization, halothane causes polyspermy (multiple sperm entry into a single egg). Dr. Hinkley demonstrated

this by direct microscopic identification of up to seven sperm in a single egg, the presence of supernumerary sperm asters in fertilized eggs, and the presence of multiple sperm nuclei in microtubule complexes isolated from eggs as early as ten minutes post-insemination. The observation is important because few, if any, polyspermic eggs subsequently undergo normal development. These results, together with data obtained earlier, show clearly that volatile anesthetics can alter embryogenesis at almost any point between fertilization and the first cell division.

"Maternal Anesthesia and the Stressed Fetus"
R01 GM 29922-02 (Shnider, S.), University of California, San Francisco
"Effects of Asphyxia on the Pharmacodynamics of Lidocaine in the Mature Fetal Lamb"
R01 GM 29571-03 (Morishima, H.), Columbia University

Fetal asphyxia due to recurrent partial compression of the umbilical cord is common during labor. However, little data exist in the literature regarding the best obstetric anesthesia to use under conditions of fetal stress. Dr. Sol Shnider has developed a model of fetal asphyxia that employs uterine artery occlusion. This model mimics closely the clinical situation when decreased uterine perfusion causes fetal distress, for example, in cases of maternal hypotension due to aortic compression, arterial vasoconstriction, uterine hypertonus or tetany. In this model, the asphyxiated fetus becomes acidotic, bradycardic, and hypertensive and redistributes cardiac output to vital organs such as the heart, brain, and placenta. He found that fetal cerebral metabolic oxygen consumption decreases with asphyxia. This decrease in cerebral oxygen consumption, coupled with preservation of cerebral blood flow, protects the most vital of all organs.

Dr. Shnider is examining the effects on the asphyxiated fetus of a variety of maternally administered anesthetic agents, including halothane, isoflurane, thiopental, ketamine and regional anesthesia. Maternal halothane administration did not significantly decrease cerebral blood flow or cerebral metabolic oxygen consumption in the asphyxiated fetus. Thus, halothane appears to be an appropriate maternal anesthetic when fetal distress is the indication for caesarean section, as it does not appear to adversely affect vital organ blood flows or cerebral oxygen consumption in the fetus.

Dr. Hisayo Morishima's research is designed to elucidate the effects of asphyxia on the fetal uptake and cardiovascular responses to maternally administered lidocaine. In oxygen-limited fetuses, Dr. Morishima found that the mean (±SE) pHa and PaO_2 fell from 7.35 ± to 0.02 to 7.23 ± 0.11, and from 22 ± 2 to 13 ± 3 mmHg ($p<0.05$), respectively, after 60 minutes of cord occlusion and was maintained throughout drug infusion. The mean lidocaine concentration in maternal blood reached a steady state of about 2.0 µg/ml after approximately 120 minutes of infusion, whether or not the fetus was oxygen-limited. In the fetus, steady state plasma concentrations of lidocaine were also reached at about 120 minutes, at about 1.0 ± 0.10 µg/ml in the control and 1.3 ± 0.17 µg/ml in the oxygen-limited fetus. Fetal tissue concentrations of lidocaine at the end of the infusion were lower than those in the corresponding maternal organs, except for the liver. Plasma concentrations of lidocaine were similar in the control and oxygen-limited fetuses, but drug concentrations were two to seven times higher in all organs of the oxygen-limited fetuses than in control fetuses. Asphyxia alone tended to

produce a mild increase in cardiac output, while the percent distribution to the brain and heart almost doubled. Drug infusion produced no further change in these parameters.

"Muscle Relaxants and Their Antagonists in Pediatric Anesthesia"
R01 GM 32202-02 (Fisher, D.), University of California, San Francisco

The duration of neuromuscular blockade produced by vecuronium, 70 g/kg, was found in an earlier study to be longer in infants (73 minutes) than in children (35 minutes) or adults (53 minutes), despite similar dose-response relationships. To explain these differences in duration of action, Dr. Dennis Fisher has determined the pharmacokinetics and pharmacodynamics of vecuronium in infants and children during nitrous oxide halothane anesthesia. Total plasma clearance was similar for infants, children and adults, but the volume of distribution at steady-state was larger, and the mean residence time longer, in infants than in children or adults. These effects are partially offset by the fact that the steady-state plasma concentration of vecuronium which causes a 50 percent twitch depression, a measure of neuromuscular junction sensitivity, is lower in infants than in children or adults. The results with vecuronium are similar to those reported earlier by Dr. Fisher for d-tubocurarine and help to explain the longer duration of action of some anesthetics in infants.

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